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Sherille Denae Bradley

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by

Sherille Denae Bradley, B.S.

APPROVED:

_____
Gregory Lizee, Ph.D.
Supervisory Professor

_____
Wei Cao, Ph.D.

_____
David McConkey, Ph.D.

_____
Dorothy Lewis, Ph.D.

_____
Stephanie Watowich, Ph.D.

APPROVED:

_____
Michelle Barton, Ph.D.
Dean, The University of Texas
Graduate School of Biomedical Sciences at Houston

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Sherille D. Bradley, B.S.

Houston, Texas, USA

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Sherille Denae Bradley, B.S.

Advisory Professor: Gregory A. Lizee, Ph.D.

The mitogen-activated protein kinase (MAPK) pathway is a cascade of serine-threonine kinases involved in cell growth, proliferation, and apoptosis; it is one of the most well-known pathways associated with melanoma progression. The MAPK pathway is constitutively activated in melanomas due to mutations in the signaling components, particularly the proto-oncogene $BRAF^{V600E}$ that accounts for (40-50%) of these cases. Metastatic melanoma is one of the deadliest and most aggressive forms of cancer, with a 3-year survival rate of less than 15%. Immunotherapies that utilize cytotoxic T lymphocytes (CTLs) have proven to be very effective at inducing regressions of large, bulky tumors, and in improving melanoma patient survival. Two MAPK targeted inhibitors, vemurafenib and dabrafenib, have produced positive results in clinical trials thus far, but they are not without limitations. Recent studies have shown that oncogene activation in tumor cells can affect the level of expression of major histocompatibility complex I molecules (MHC-I) on the cell surface, and potentially allow melanoma cells to escape immune system surveillance. MHC-I molecules expressed by tumor cells are the crucial targets recognized by CTLs to kill tumor cells; thus, strategies to improve MHC-I antigen presentation to T-cells is very likely to improve the efficacy of current immunotherapies. In DCs and other hematopoietic cell types the rapid internalization and recycling of MHC-I through endocytic compartments has been characterized as a cytoplasmic tail dependent process. The MHC-I cytoplasmic tail

possesses two conserved phosphorylation sites, a tyrosine (Y320) and serine (S335). It is known that MAPK pathway activation can induce a phosphorylation signaling cascade in tumor cells, but the mechanism behind the regulation of MHC-I internalization and modulation has yet to be identified in tumor cells.

In our study we show that both BRAF and MEK inhibitors increase MHC-I surface expression in $BRAF^{V600E}$ melanoma mutant cell lines. Additional studies have shown that the level of MHC-I surface expression directly affects CTL recognition and cytokine release. We also reveal a novel potential mechanism for MAPK pathway regulation of MHC-I through the highly conserved serine encoded by exon 7 of the MHC-I cytoplasmic tail. These studies suggest that the mechanism behind MAPK regulation of MHC-I is through serine phosphorylation and inhibition of this process allows for a longer surface half-life of MHC-I molecules leading to better CTL responses. Ultimately, we have shed light on MHC-I surface expression, trafficking, internalization and antigen presentation in melanoma. Knowledge gained through this study could aid in the development of cancer treatment strategies whereby MAPK pathway inhibition is used to augment the effectiveness of CTL-based immunotherapies. Furthermore, these types of therapeutic approaches may be generalizable to other tumor types that also demonstrate constitutive MAPK pathway activation.

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Chapter I. Introduction and background

ABBREVIATIONS

MHC: Major Histocompatibility Complex

HLA: Human leukocyte antigen

CTLs: Cytotoxic T Lymphocytes

TILs: Tumor infiltrating lymphocytes

DCs: Dendritic cells

APCs: Antigen presenting cells

MAPK: Mitogen activated protein kinase

ERK: Extracellular-signaling regulated kinase

BRAF: Serine/threonine-protein kinase B-Raf

BRAFi: BRAF inhibitor

NRAS: Neuroblastoma RAS

PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase

AKT: Protein Kinase B

mTOR: Mechanistic target of rapamycin

GTP: Guanine Tri-phosphate

GDP: Guanine Di-phosphate

MART-1: Melanoma antigen recognized by T-cells 1

IL-2: Interleukin-2

PD1: Programmed cell death protein 1

PDL1: Programmed death ligand 1

CTLA-4: Cytotoxic T- lymphocyte antigen-4

VEGF: Vascular endothelial growth factor

CHAPTER I:

INTRODUCTION AND BACKGROUND

1.1 MELANOMA

Melanoma is a dangerous form of skin cancer that often develops from melanocytes exposed to UV radiation and the body's inability to repair the damaged cells. Unrepaired DNA damage leads to mutations within the melanocytes that can transform the cells into cancerous cells. Melanoma is known to metastasize to other parts of the body. Metastatic melanoma is one of the deadliest and most aggressive forms of cancer, with a 3-year survival rate of less than 15% [1]. Immunotherapeutic approaches that utilize cytotoxic T lymphocytes (CTLs) have proven to be very effective at inducing regressions of large, bulky tumors, and in improving melanoma patient survival [2].

Proto-oncogenes are genes that are known to a have high potential to induce cancer. The most relevant proto-oncogene in metastatic melanoma is BRAF kinase, a component of the mitogen-activated protein kinase (MAPK) pathway. Constitutive activation of this pathway by a characteristic BRAF mutation (V600E) contributes to the fatality of metastatic melanoma by inducing many uncontrolled cellular changes, including cell proliferation and immune suppression. Current oncogene- targeted approaches have helped to extend the life of many patients, but they have generally not proven to be effective in extending the lives of patients beyond 6 or 7 months. With a better understanding of how the MAPK pathway is affecting the immune response, we can develop strategies to improve the efficacy of current melanoma therapies.

1.2 MAP KINASE SIGNALING PATHWAY

Protein kinases are enzymes that phosphorylate proteins by covalently attaching phosphate groups to the side chain of serine/threonine or tyrosine residues [3], and regulate the activity, localization, and function of proteins involved in many cellular processes. Kinases most commonly are involved in signal transduction and cell cycle regulation. There are many of distinct protein kinases, and in recent years over 100 different kinases have been discovered [4]. Based on their substrate specificity, kinases fall within certain family groupings. Kinases can be characterized as serine/threonine or tyrosine kinases.

This study specifically focuses on serine/threonine kinases, which play a major role in the control of the cell cycle and in receptor-mediated signaling pathways [5]. Mitogen-activated protein kinases (MAPK) are a group of highly conserved serine/threonine protein kinases, whose function and regulation have been conserved throughout evolution, from simple organisms like brewer's yeast to highly complex organisms, such as mammals [3]. Years of research have been invested to characterize the protein kinases involved in this pathway. Despite the extensive characterization of the signaling cascade, there are still many unknowns surrounding the downstream effects of this pathway on gene expression. Because this signaling pathway is often deregulated in tumor cells, a more complete understanding of this signaling pathway could lead to improvements of targeted-therapies for cancer patients.

The MAPK pathway specifically consists of the Ras/Raf/MEK/ERK (extracellular-signal-regulated kinase) pathway, a series of protein kinases that regulate the expression of proteins involved in cellular growth and survival (**Figure 1.1)** [6,7]. The MAPK pathway responds to the binding of cytokines, growth factors, hormones, and other stimulating

factors that bind to receptors on the cell surface [6,7,8]. Because of their important roles in cellular functions , MAPK components have been studied extensively in human diseases.

MAPKs are responsible for catalyzing the addition and removal of phosphates to substrate proteins; as such, they act as an on and off switch to control the activity of other proteins [3]. The process of phosphorylation is regulated by the feedback loop system of MAPKs and protein phosphatases working in a reciprocal matter to change the behavior of cells in response to extracellular signals. There are three central, sequentially-activated kinases that make up the signaling cascade in the MAPK pathway, these three kinases are known as MAPKKKs (ex. BRAF), MAPK kinase (ex. MEK 1/2), and MAPK (ex. ERK 1/2). These kinases are all serine/threonine kinases that are activated through phosphorylation and their interaction with GTP proteins of the Ras/Rho family in response to extracellular stimuli [5,9].

Our study will focus on the ERK 1/2 pathway, one of major MAPK signaling pathways [10]. The ERK 1/2 pathway was the first pathway identified and currently the best described MAPK pathway [10]. The ERK cascade typically responds to extracellular growth signals. In response to stimuli, the Ras-guanosine diphosphate (GDP) is exchanged with Ras-guanosine triphosphate (GTP) which binds to Raf, leading to the activation of kinases in the pathway. The binding of RasGTP to Raf family members relays a signal to the MAPK signal cascade; once the MAPKs are activated, they phosphorylate serine/threonine residues on their target proteins [10].

There are three Raf proteins expressed by human cells: RAF-1, ARAF, and BRAF [7]. The focus of this study is the raf protein BRAF. When BRAF is activated, it

phosphorylates the kinases MEK 1/2, which in turn activates downstream phosphorylation in the MAPK pathway. In normal cells, the activation of the MAPK pathway is quickly reversed and the MAPK phosphorylation cascade is switched off [11]. One of the major hallmarks of cancer is the ability of cancer cells to sustain their proliferation signals. Mutations that induce the constitutive activation of MAPK pathway components can induce uncontrolled cellular proliferation, which will be further outlined in the following section.

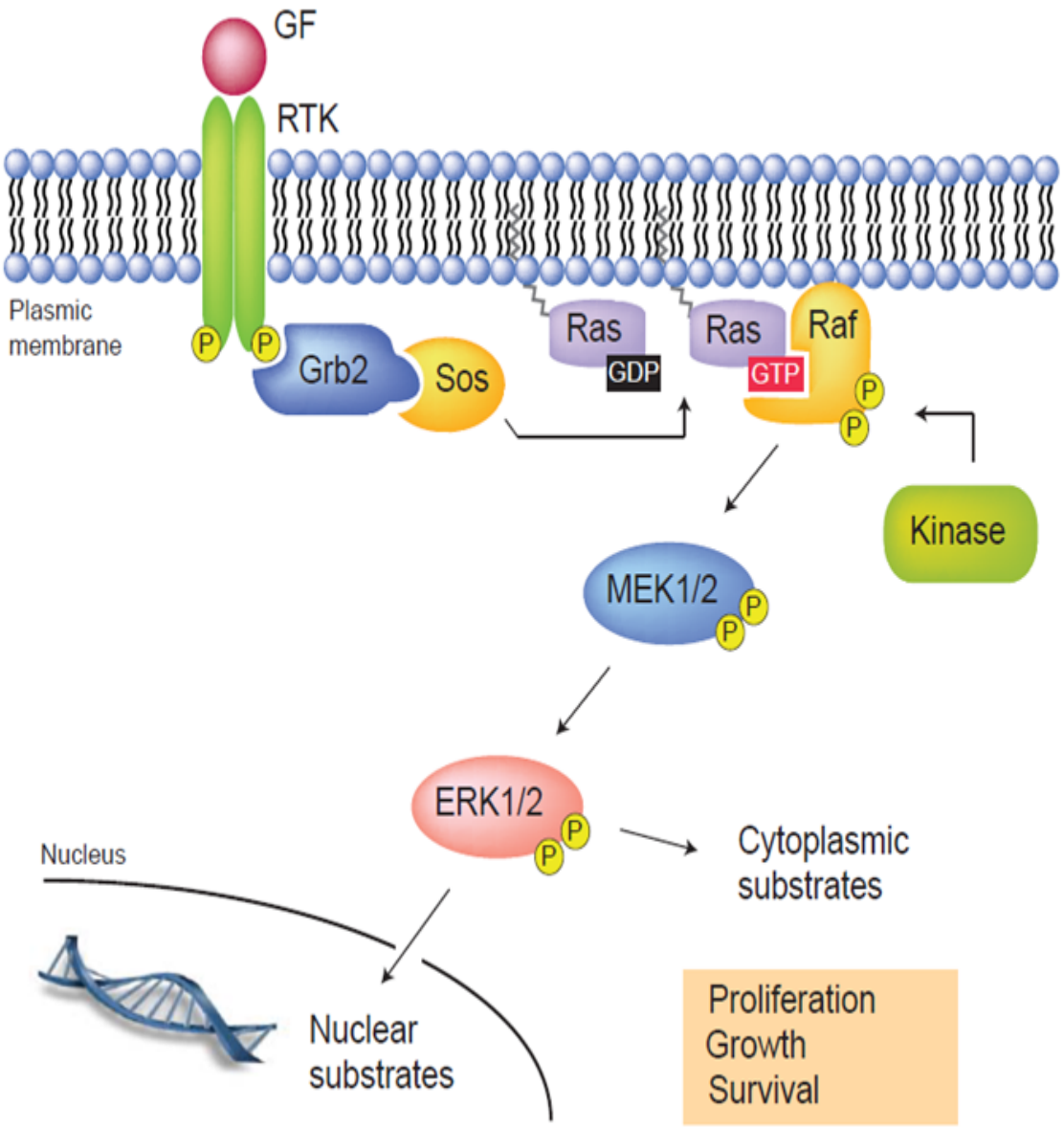


Figure 1.1 Schematic representation of the MAP kinase pathway. MAP kinase pathway schematic representation showing sequential kinase phosphorylation and activation. [12] C. Fremin, S. Meloche, From basic research to clinical development of MEK1/2 inhibitors for cancer therapy, J Hematol Oncol 3 (2010) 8. (*Reprinted with permission of the authors).*

1.3 MAP KINASE PATHWAY SIGNALING BY ONCOGENES

Proto-oncogenes are genes that are activated by changes in normal DNA such as mutations, translocations, or gene amplifications resulting in increased function for that individual gene [13,14]. These genetic events lead to its constitutive activation and dominance over the normal functioning gene. The pathogenesis of malignant tumors can be largely attributed to the constitutive activation of oncogenes within the tumor cell. Activating point mutations can lead to the uncontrolled cell proliferation, as described for the RAS and RAF oncogenes [15,16]. RAS and RAF are components of the MAPK kinase signaling cascade, and mutations to these proteins RAS or RAF have been identified in multiple forms cancer with varying degrees of prevalence. Such mutations have been identified in melanomas, colorectal cancers, lung cancers, sarcomas, liver, and breast cancers [11].

In melanoma, the MAPK pathway predominantly activating contains mutations in the NRAS and BRAF proto-oncogenes. NRAS mutations account for 15-20% and BRAF mutations account for 40-50% of cases of constitutive activation of this pathway [11,13,17]. Over 90% of BRAF mutations consists of a single substitution of thymine with adenine at nucleotide 1799; this change leads to valine (V) being substituted by glutamate (E) at codon 600, and the mutation is referred to as $BRAF^{V600E}$ [18]. Immunosuppression has recently been linked to the activation of the MAPK signaling pathway thru $BRAF^{(V600E)}$ mutations. For example, the oncogenic MAPK pathway upregulates the inflammatory cytokines IL-1 α/β in melanoma cells [19]. Tumor-associated fibroblasts (TAFs) in turn respond to IL-1 upregulation by producing PD-1 ligands, and COX-2, leading to an ongoing suppression of CTLs preventing them from performing their function [20]. In addition to the suppression of

the immune response, BRAF mutations are also associated with a poorer prognosis for these patients.

The bridge between CTL-mediated immunotherapy and other therapies that target the BRAF $^{(V600E)}$ mutation has grown tremendously, with a wide array of new developments in many areas, including combination therapies. As discussed below, there is growing evidence that such combinations may act in synergy to improve response rates for melanoma patients.

1.4 BRAF-TARGETED THERAPY AND CLINICAL RESULTS

Each year in the U.S., 76,000 new cases of melanoma are diagnosed, and 9000 people died from the disease in 2012 [21]. The aggressive nature of melanoma, once it spreads, has been a major issue for the treatment of patients. Early diagnosed cases of melanoma can be surgically removed, but not all tumors are resectable. In advanced stages of melanoma, chemotherapy is not very effective due to the rapid development of resistance. Furthermore, metastatic melanoma proliferates at a rapid pace and can disseminate to almost any part of the body [21]. Prognosis for metastatic melanoma patients is poor, with only 10% of patients living to 5 years post diagnosis, and the median survival being less than a year [22].

Many different therapeutic approaches have been taken to treat metastatic melanoma patients. The FDA-approved chemotherapy for melanoma treatment Dacarbazine induces objective responses in only 10-15% of patients, but has no benefits for on progression-free or overall survival of patients. The use of drugs that activate CTLs, like interleukin-2, yield higher rates of complete responses, but do not result in a higher overall survival rate [23]. In

recent years, the focus has turned to the use of oncogene-targeted therapies to provide a more effective treatment for melanoma sufferers. BRAF-targeted inhibitors have become the new standard of treatment for aggressive melanomas, but issues with tumor resistance have plagued clinicians and researchers since the development of these drugs. The need to gain more knowledge, and better understand how resistance in the MAP kinase pathway drives tumor progression remains paramount to the development of better treatments for melanoma.

Dozens of small-molecule inhibitors have been developed over the past couple of decades to target specific mutated oncogenic proteins within the MAP-kinase pathway **(Table 1.1)** and clinical trials have been designed to test these inhibitors. In 2002, the first BRAF mutations were reported. At that time, Sorafenib was the best available kinase inhibitor. Sorafenib can inhibit a broad spectrum of cellular targets, such as VEGFR-2, PDGFR, and C-KIT, FLT-3, CRAF, wild-type BRAF or $BRAF^{V600E}$ [24]. Sorafenib was able to inhibit BRAF but in order to work for MEK and ERK deactivation, high micromolar concentrations were required that led to off-target effects and toxicities [25]. Therefore, a need for more selective oncogene-targeted inhibitors led to the development of BRAF inhibitors that specifically target mutated BRAF (V600E).

BRAF (V600E) -targeted therapy is one of the most significant therapeutic advances for the treatment for melanoma in many years. PLX-4032 (Vemurafenib) was the first BRAF inhibitor approved by the US Food and Drug Administration (FDA) for treatment of patients with metastatic melanoma or unresectable tumors [26]. GSK2118436 (Dabrafenib) is another BRAF inhibitor that also has been approved by the FDA. Because both inhibitors are specific for the mutated BRAF, they are classified as type I BRAF kinase inhibitors

[21]. Although both inhibitors can act on WT BRAF at higher concentrations, they have both induced significant beneficial effects on patients in clinical trials thus far [25].

The first human trials using vemurafenib and dabrafenib were performed in metastatic melanoma patients [27]. In the vemurafenib phase I clinical trial, 32 patients enrolled and 81% of the enrolled patients possessed a BRAF $^{(V00E)}$ mutation. Each patient received the maximum dosage of 960mg twice a day, and both complete and partial responses were recorded [28]. Phase II and phase III trials confirmed the results from the phase I trial, and showed about 48% of the patients responded to treatment [29]. Similar response rates were observed in a parallel dabrafenib phase I trial. In the first dabrafenib trial, patient tumors were measured at baseline and two weeks after the start of BRAF inhibitor treatment. The level of ERK phosphorylation was also analyzed at each point. In patients that responded, 80% showed phospho-ERK inhibition and some patients had inhibition levels as high as 90-95% [25]. Patient response rates varied from 2 months up to over 2 years of progression-free survival, but the median duration of response was roughly 7 months.

By contrast, the MEK inhibitor GSK1120212 (Trametinib) is an example of a selective small-molecule MEK 1/2 inhibitor [22]. In a phase III trial, a total of 322 patients with metastatic melanoma were randomly treated with either trametinib or one of two chemotherapy drugs. The overall goal of the trial was to get all patients to progression-free survival; if this did not occur for patients treated with chemotherapy, they had the option to switch to trametinib treatment. The median progression-free survival of trametinib-treated patients was 4.8 months and for the chemo-treated patients it was 1.5 months. At 6 months, the overall survival was 81% for trametinib patients and 67% in the chemo treated group. At

the end of the trial, 22% of patients in the trametinib-treated group had objective responses compared to only 8% of the chemotherapy treated group [22 ,25].

With all three MAP kinase inhibitors, toxicity and side effects can be a threat to progression-free survival. Both vemurafenib and dabrafenib caused rashes and the development of squamous-cell carcinomas in some patients that was attributed to the appearance of HRAS mutations [30]. The carcinoma lesions that appeared in some patients were largely managed by excision [30,31]. As for patients treated with trametinib, the side effects experienced were more severe, ranging from rashes to diarrhea, and peripheral edema [32] [33].

Table 1.1 Small Molecule Inhibitors Used for Targeting MAPK Pathway.

MAPK Pathway Target	Inhibitors
RAS	Tipifarnib, Lonafarnib, Salirasib, BMS-214662, L-778123
BRAF	Sorafenib, Dabrafenib, Vemurafenib, GDC-0879,
MEK	Trametinib, Selumtinib, Refametinib, MEK162
ERK	AEZS-131, SCH772984

BRAF-targeted therapy has provided many patients with increased progression free-survival time, but the development of resistance has been a major problem associated with treatment. The MAP kinase pathway can contain multiple mutations that activate ERK, and

some patients develop alternative mutations (i.e. NRAS) that are not specifically targeted by BRAF inhibitors. In clinical trials, it was determined that at least two-thirds of patients developed restored ERK phosphorylation despite prior inhibition [25]. The activation of ERK signaling took place as early as two weeks into treatment, and in some cases the amount of ERK phosphorylation was increased in comparison to baseline levels of phosho-ERK [25]. Resistance mechanisms include NRAS mutations, alternatively-spliced BRAF variants, amplification of BRAF and downstream changes to MEK activation [34].

To combat resistance, new potential targets for the MAP kinase pathway have been identified. Different MEK inhibitors could possibly decrease or prevent the occurrence of pERK re-emergence post BRAF inhibitor treatment. MEK targets that are downstream of RAF may be good potential targets. In addition the Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), Protein kinase B (AKT), mechanistic target of rapamycin (mTOR) (better known as the PI3K/AKT/mTOR) pathway is involved in melanoma signal transduction; phosphatase and tension homolog (PTEN), a suppressor gene in the pathway, suggest potential additional targets to stop the reactivation of downstream ERK. Pre-clinical data support the possibility that these pathways are relevant to the pathogenesis of BRAF-mutant melanoma and could possibly be used by tumor cells as a means for immune evasion [35] [36].

An alternative approach to treating resistance in metastatic melanoma patients is the use of combination therapy. For example, in addition to combining targeted agents like BRAFi and MEKi, more recently the combination of targeted agents with checkpoint inhibitors, such as ipilimumab, a monoclonal antibody that blocks the co-inhibitory molecule cytotoxic t-lymphocyte associated protein 4 (CTLA-4), has gained more traction

clinically [37]. Ipilimumuab was approved by the FDA in 2011 and is currently undergoing clinical trials for use in combination with immunotherapies for melanoma patients. In addition to the use of ipilimumuab, newly developed monoclonal antibodies that block programmed cell death 1 (PD-1) and its ligand, CD274 (PD-L1) are also being tested currently. As always, many questions arise as to how these combinations will affect patient tumor progression, survival and overall health. Regardless, the future of BRAF-targeted therapy and immunotherapy as means of treatment for metastatic melanoma patients looks promising, as outlined further below.

1.5 CTL-MEDIATED IMMUNOTHERAPY

Cancer immunotherapies have grown exponentially in recent years; where chemotherapies have failed, they have proven to be a respectable alternative treatment. The adaptive immune response relies heavily on immune effector cells known as cytotoxic t-lymphocytes (CTLs). Also referred to as CD8+ T-cells, CTLs are known to be major players in the regression of tumors. CD8+ T-cells recognize major histocompatibility complex class I (MHC-I) molecules that display peptide antigens on all cell types in the body. In addition to distinguishing self-antigens and non-self-antigens, effector CD8+ cells can be primed to target antigens that are located on different types of cancers, known as tumor associated antigens (TAAs) [38]. Lymphocytes that infiltrate into the stroma of cancer nodules are referred to as Tumor infiltrating lymphocytes (TILs) [39,40]. TILs provided the first evidence that the immune system can recognize tumor antigens [41,42]. In 1988, Wolfel et al. showed that target structures located on human leukocyte antigens (HLA) displayed on tumor cells could induce the killing of these cells by CTLs [42]. The target structures identified were later described as the first tumor antigens recognized by T-cells [43].

Intracellular proteins are comprised of poly-peptide chains, and within a cell they are split into shorter peptides that can be presented at the cell surface when loaded onto MHC-I molecules. Peptides can range from 8 to 12 amino-acids that are derived from specific cell-associated proteins, and are recognized by the T-cell receptor of CTLs [44]. Peptides can only bind to specific MHC alleles that possess particular amino acid binding motif preferences [44,45]. The targeting of TAAs through various treatment methods has been the foundation of cancer immunotherapy.

Several different T-cell based immunotherapies have been developed to treat melanoma. For example, the cytokine interleukin-2 (IL-2) is responsible for the growth, proliferation, and differentiation of T-cells to become effector cells. IL-2 is produced by T-cells following T-cell recognition of a foreign pathogen or a tumor antigen. IL-2 as monotherapy or combinations of IL-2 and TILs were some of the first successful T-cell mediated immunotherapies developed for the treatment of cancers [39]. Cancer vaccines can also be used to prime anti-tumor T-cell responses. Melanoma researchers have identified proteins that are expressed with high frequency amongst most melanoma patients, Melan-A/MART-1 and gp-100. Because these proteins are expressed only in melanocytes and melanoma cells, they are considered good TAA candidates [44,46]. Certain cancer vaccines utilize peptides derived from these proteins to induce an adaptive immune response. Tumor antigens combined with an adjuvant can be used to prime the immune system to develop more antigen specific T-cells and more potent anti-tumor immunity. However, the use of vaccines for activating T-cells is not without complications, under certain circumstances peptide-specific T-cells can become tolerized and become unresponsive [47]. This can range from issues with the dose of peptide or the adjuvant used during immunization. An over-

stimulation of the immune response can also lead to exhaustion of memory T-cells within the body.

An alternative to peptide vaccines involves using them in conjunction with professional antigen presenting cells (APCs) that have been loaded with peptides and injected into patients. The most potent APCs for inducing T-cell activation are dendritic cells (DCs); T-cell receptors interact with MHC-I on DCs, leading to T-cell priming. DCs normally internalize protein antigens from cells, process the antigens and then travel to the lymph nodes to present the peptides to T-cells [48]. APCs also have the unique ability to present both endogenously derived peptides through MHC-I and exogenously derived peptides on MHC-II complexes. In a process known as cross presentation, APCs can present also exogenously-derived peptides on recycled MHC-I molecules [49]. The discovery that DCs normally uptake antigens and present them to activate T-cell responses led to the rationale that peptide-loaded DCs would be good candidates for initiating an anti-tumor response [49,50,51]. Although most DC-based cancer vaccines have shown promise for inducing anti-tumor T-cells in peripheral blood, induction of clinical responses has been comparatively rare.

1.6 ROLE OF MHC-I IN T-CELL MEDIATED IMMUNE RESPONSES

MHC-I is a central focus of immune response. Peptides derived from many types of cells including self, non-self, pathogen, and tumor-derived are displayed by these molecules to effector T-cells by all nucleated cells of the body. MHC-I molecules are able to present endogenously-derived peptides, but in professional antigen presenting cells (APCs), such as dendritic cells (DCs), MHC-I molecules are also able to present exogenously-derived

peptides. Mature MHC-I complexes are assembled in the endoplasmic reticulum (ER); they are heterodimers assembled from a polymorphic heavy chain, and a light chain called β2-microglobulin (β_2m) and antigenic peptide [52,53]. Since they display these peptides at the cell surface, and MHC-I molecules play an important role in antigen presentation. Proteasomes degrade cytoplasmic proteins into smaller peptides, which are transported into the ER by the transporter associated with antigen presentation (TAP) and are loaded in to the peptide binding grooves of the MHC-I molecules. The MHC-I binding groove accommodates peptides 8 to 12 amino acids in length [52]. In the ER, MHC-I molecules are stabilized by chaperone proteins such as calreticulin [54]; in addition, the molecule Tapasin interacts with TAP to assist in the delivery of peptides to MHC-I molecules. When a peptide is successfully bound to MHC-I and β2- microglobulin as a trimolecular complex, it exits the ER via the secretory pathway and travels to the cell surface in vesicles for presentation.

Human MHC-I molecules are referred to as human leukocyte antigens (HLA). The three classical MHC-I genes are HLA-A, HLA-B, and HLA-C, and each HLA type can recognize and bind uniquely to certain peptides because of the molecular properties of the their peptide binding grooves [55]. HLA-A and HLA-B are expressed in higher levels in the human cells than HLA-C. The extremely high level of MHC-I Polymorphism results in different peptide-binding grooves that recognize and bind characteristic peptide sequences, allowing for each individual to present a wide and distinct array of peptides.

Each HLA molecule loaded with peptide is expressed on the cell surface to present peptides to CD8+ T-cells, this can induce clonal expansion of effector of T-cells, target cell killing or cytokine release, depending on the APC and T-cell differentiation state. T-cells

contain a T-cell receptor that binds to MHC-I; when this occurs; many more stabilizing receptors bind to one another from each cell thus allowing the T-cell to stay in close contact to release effector molecules. Granzyme B and perforin are two common molecules released by CD8+ cytotoxic T-cells to induce target cell death. Granzyme B is a serine protease secreted by T-cells (and other non-cytotoxic cells like mast cells and basophils) that is involved in inducing inflammatory cytokine release [56]. Perforin is a pore-forming protein that enters target cells; once inside, it triggers an increase of intracellular calcium and eventually leads to the apoptosis of target cells [54].

MHC-I molecules possess both an extracellular region that contains the peptide-binding region, a transmembrane domain, and a cytoplasmic tail. The MHC-I cytoplasmic tail plays a role in antigen presentation, but it has not been extensively studied. Studies have shown that the tail is involved in intracellular trafficking and antigen-presentation in APCs, but little is known about its role in tumor cells. The MHC-I cytoplasmic tail is 35 amino acids in length and is encoded by three exons: 6, 7, and 8. This region is highly conserved throughout species [52] [57]; Exon 6 and 7 each contain potential tyrosine and serine phosphorylation sites, respectively [53]. Recent research has revealed that a natural splice variant occurs in some organisms (not in human beings); whereby exon 7-encoding amino acids are deleted from cytoplasmic tail region. Previously published work from our lab revealed that DCs transduced with this exon 7-deleted variant had a longer cell surface half-life and this contributed to better recognition and killing by T-cells [57].

Furthermore, previous studies showed that tyrosine-320 encoded by exon 6 is crucial for MHC-I endolysosomal trafficking in DCs and contributes to the ability of MHC-I to acquire and present exogenously derived peptides [53]. MHC-I molecules utilized by DCs

are extremely important for activating T-cell mediated adaptive immune responses. The fact that the MHC-I cytoplasmic tail plays a role in trafficking in DCs may also help shed light on the role of the cytoplasmic tail in antigen presentation by tumor cells or other nucleated cells, which will be discussed further below.

1.7 MODULATION OF MHC-I BY PATHOGENS AND ONCOGENES

Since MHC-I displays fragments of intracellular proteins on the cell surface, it is essential for immune surveillance by T-cells. The MHC-I molecule is expressed by all nucleated cells; any cell can be infected by a viral pathogen, or subjected to mutations that may lead to cancer. Peptides derived from mutated proteins are presented to CD8+ T-cells, which in turn can induce an adaptive immune response. Interestingly, pathogens and cancer cells evolve ways to avoid being detected by the immune system in order to downgrade the immune response as much as possible. Since MHC-I molecules are so fundamental to the immune response, they are often the targets of these mechanisms of immune evasion. Defects in MHC-I mediated antigen presentation renders cells unrecognizable to T-cells and provide an important advantage to both pathogens and tumor cells.

Several different components of the antigen presentation machinery (APM) can be lost or targeted, which can have implications for MHC-I mediated antigen processing, loading, and presentation. For example, defects in the proteasome that cleaves proteins into peptides have been identified in some tumor cell types. In addition, downregulation of subunits of the proteasome such as β, MB1, and Z have been characterized in carcinomas, colorectal, ovarian and bladder cancers [58,59,60]. In melanoma cells, downregulation of other inducible subunits (LMP2, 7, and 10) related to the proteasome have been

characterized [61,62]. Without proteasomes, cells can't produce the peptides required for MHC-I surface expression and T-cell killing of target cells. Other APM components commonly lost in tumor cells are the TAP1 and TAP2 genes. Tumor cells have been found to downregulate these peptide transporters, which are important for loading peptides onto MHC-I molecules [58]. In addition, viruses such as HCMV can express proteins that directly inhibit TAP 1 and 2.

The MHC-I cytoplasmic tail is also a target of viruses within tumor cells. Human immunodeficiency virus (HIV) is one of the most deadly diseases that can affect humans, partially due to its ability to escape the immune system. HIV encodes a molecule called Nef, which targets the MHC-I molecule at the cytoplasmic tail, thus disrupting the trafficking and intracellular localization of MHC-I [63]. Nef was shown to associate with the cytoplasmic tail in humans, in a manner that was dependent on the tyrosine-320 residue encoded by exon 6 [63]. When the cytoplasmic tyrosine of the HLA-A2 molecule was mutated, Nef lost the ability to downregulate the surface expression of MHC-I [64].

Sometimes tumor cells can overexpress growth factor receptors, leading to the downregulation of MHC-I molecules as a mechanism of immune system escape. For example, expression of HER2 in breast cancer cells has been shown to inversely correlate with MHC-I expression. HER2 is a proto-oncogene and a member of the epidermal growth factor (EGFR) family of tyrosine kinases. A study conducted by Inoue et al. showed that the overexpression of HER2 led to a downregulation in HLA-A2 molecules and less recognition by HER2-specific CTLs [65]. Interestingly, mutated HER2 overexpression is also linked to activation of the MAPK pathway [66].

Other oncogenic mutations have been shown to regulate the level of MHC-I on the cell surface. In particular, BRAF inhibitors (BRAFi) have been shown to induce an upregulation of MHC-I, melanoma differentiation antigens, and other immune system activators [19]. When metastatic melanoma cells were treated with BRAFi PLX 4720 (vemurafenib) in conjunction with IFN-γ, nanomolar concentrations of vemurafenib enhanced MHC-I, MHC-II, and β2- microglobulin in Mel A375 cells after 24 hours [67]. This upregulation of MHC- I has also been seen in other melanoma studies. For example, MAP kinase pathway inhibition induced upregulation of MHC-I and melanoma antigens, MART-1, gp100, and trp-1/2 in multiple melanoma cell lines after 72 hours of drug treatment [68].

Notably, BRAF inhibition has been shown in several studies to relieve immune suppression within the melanoma tumor microenvironment, leading to better CTL infiltration and function. In vitro experiments have shown that BRAF inhibition can decrease the release of immunosuppressive cytokines such as, IL-1, IL-6 and IL-8 [19]. In vivo mouse studies have also shown that PLX4720 (BRAFi) can significantly increase tumor infiltration of adoptively transferred T-cells, and induce better antitumor activity through the inhibition of melanoma tumor cell production of vascular endothelial growth factor (VEGF) [69]. In addition, melanoma patient tissue samples examined after BRAFi treatment showed an increase in CD8+ T-cell infiltration and markers of T-cell cytotoxicity. This increase of CD8+ cells was attributed to a decrease in the immunosuppressive cytokines IL-6 and IL-8 [70].

An increase in antigen presentation following MAP kinase pathway inhibition appears to be one outcome of BRAF inhibitor treatment, but it remains unclear how MHC-I expression is modulated by the MAP kinase pathway at the mechanistic level. This is important, since MHC-I upregulation following treatment by MAPK inhibitors should lead to better CTL recognition and killing of melanoma cells. How exactly is the MAP kinase pathway connected to MHC-I surface expression? A better understanding of how MAPK pathway and MHC-I are all connected may help shed light on a potentially important immune system evasion mechanism that tumor cells use. The rationale behind this project is that MHC-I down regulation on the cell surface of melanoma cells, can be reversed by treatment with MAPK pathway inhibitors. This occurrence has been documented in melanoma [65,67,71]. Increases in MHC-I surface expression following inhibitor treatment suggest that the upregulation is regulated by MAPK signaling. Since the MAPK pathway induces a phosphorylation cascade, we explored the hypothesis that MHC-I cytoplasmic tail phosphorylation mediates this connection. A potential model for how this connection might be mediated is shown in **Figure 1.3**.

1.8 AIMS OF PROJECT

It is our central hypothesis that MAP kinase pathway activation upregulates MHC-I surface expression through altering phosphorylation of the MHC-I cytoplasmic tail. The goal of this project is to identify the mechanism behind the regulation of MHC-I surface expression, which has yet to be identified in tumor cells [67].

To determine the connection between the oncogenic MAPK pathway and the surface expression of MHC-I, we explored two aims:

AIM 1: Assess the effects of MAPK pathway inhibitors on MHC-I surface expression in melanoma cells and their subsequent recognition by melanoma antigen specific T-cells.

- ***Aim 1.1.*** Determine the effects of three different clinically used BRAF / MEK inhibitors on total MHC-I cell surface expression with a panel of human melanoma cells.

- ***Aim 1.2*** Determine the role of the MHC-I cytoplasmic tail in the MAP kinase pathway dependent modulation of MHC-I surface expression in melanoma cells.

- ***Aim 1.3***. Determine if modulating the level of melanoma cell MHC-I surface expression through MAPK pathway inhibition improves CTL-mediated recognition and killing.

AIM 2: Identify the mechanism of modulation of MHC-I molecules by the oncogenic MAPK pathway.

- ***Aim 2.1*** Determine the steady state rate of internalization of MHC-I molecules in BRAF mutant melanoma cell lines.

- ***Aim 2.2:*** Determine whether MAPK pathway inhibitors slow the rate of internalization of MHC-I molecules, and if the process is cytoplasmic tail dependent.

This research is significant because MHC-I molecules are crucial targets recognized by CTLs to kill tumor cells; thus, strategies to improve MHC-I mediated antigen presentation to

CTLs are likely to increase the efficacy of current immunotherapies. Knowledge gained from these experiments can ultimately improve our understanding of how the MAPK pathway controls MHC-I surface expression, trafficking, internalization, and antigen presentation in melanoma. A better understanding of how oncogenic signaling pathways regulate MHC-I may allow for the development of novel cancer treatment strategies whereby different inhibitors targeting MHC-I downregulation can be used to augment the effectiveness of CTL-based immunotherapies. Furthermore, these types of therapeutic approaches may be generalizable to the many tumor types that demonstrate constitutive MAPK pathway activation.

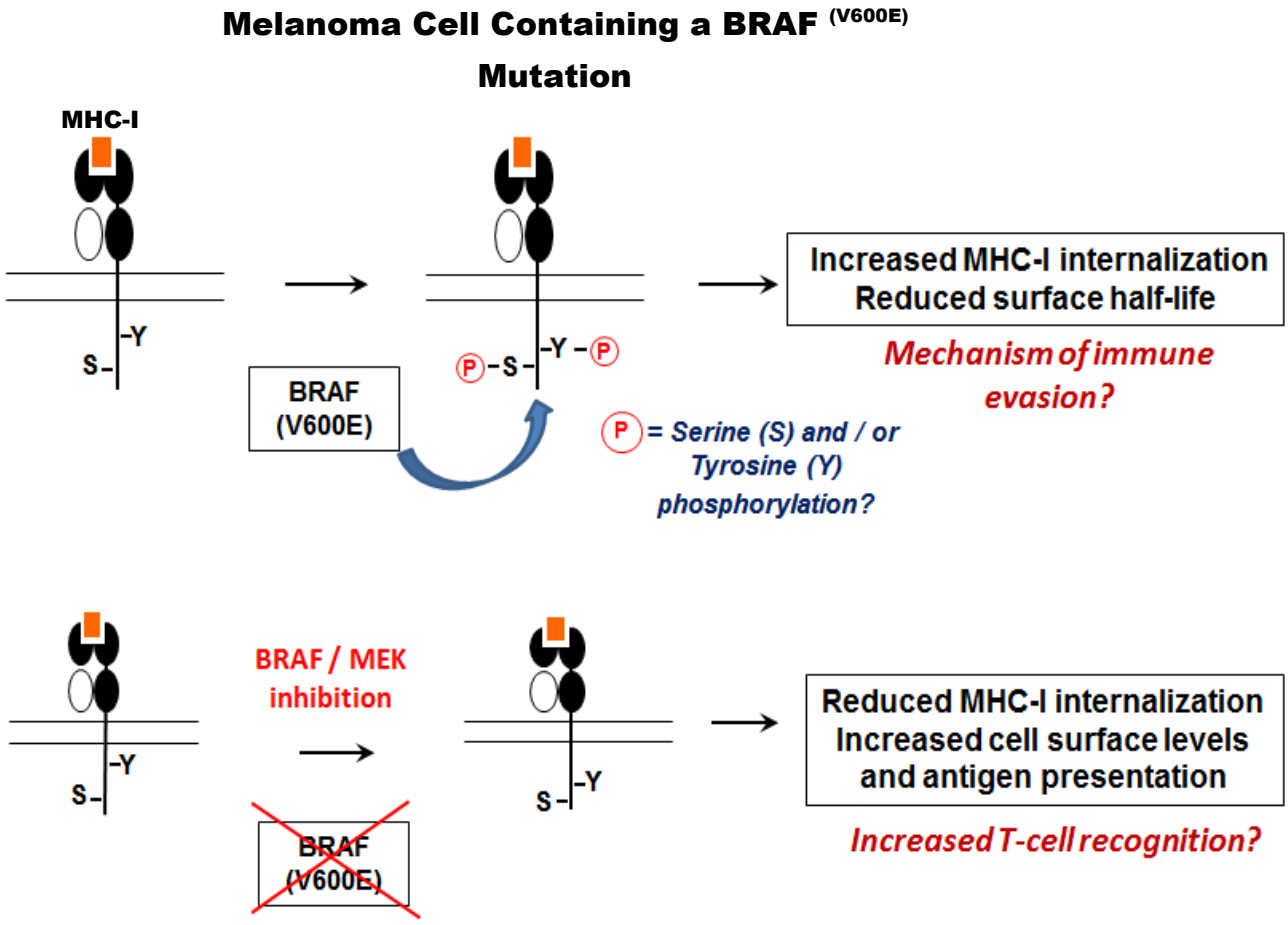


Figure 1.3. Potential role of MHC-I tail phosphorylation in controlling MHC-I trafficking in melanoma cells. Working model of possible mechanism of the MHC-I cytoplasmic phosphorylation before and after BRAF inhibition in melanoma cells.

CHAPTER II:

MATERIALS AND METHODS

2.1 Cell Culture and Transduction

Human melanoma cell lines expressing $BRAF^{V600E}$ mutations, Mel888 and WM793 were transduced with HLA-A*0201 isoforms using lentiviral gene transfer vectors (**Figure 3.2 A**). The human (hPGK) promoter was used to drive the expression of WT, ΔT, ΔS and ΔY HLA-A*0201 isoforms [57]. Transduced cells expressing similar levels of surface HLA-A2 were isolated by cell sorting (**Figure 3.2 B**). Additionally, three untransduced human melanoma lines were utilized, two BRAF-WT cells HS294T, Mewo and Melanocytes. Mel888 lines were cultured in RPMI 1640 medium (GIBCO Grand Island, NY) containing 10% fetal bovine serum (GIBCO), 10 IU/mL penicillin (Cellgrow Manassas, VA), 10 µg/mL streptomycin (Cellgrow), Insulin-Transferrin-Seleum-A (GIBCO) and maintained at 37°C in 5% CO_2. WM793, HS29T, and MeWo cells were grown in similar media without the addition of insulin. Melanocytes were cultured in HEMa media (Life Technologies). TILs were utilized in T-cell assay experiments. TIL were maintained in RPMI-1640 medium containing 10% fetal bovine serum, 10 IU/mL penicillin, 10ug/mL streptomycin and supplemented with 200 IU/mL of IL-2.

2.2 Isolation and REP of TIL

MART-1 CD8+ specific tumor infiltrating lymphocytes (TIL) obtained from infusion product (>98% MART-1 specific) were generously donated by Dr. Laszlo Radvanyi (M.D. Anderson Cancer Center Houston, TX). To obtain TILs, melanoma tumor cells were resected from stage IV- melanoma patients. The tumors were cut into 3-5mm^2 fragments. The fragments were placed in a 24 well plate with TIL culture media (TIL-CM) and 6000 IU/ml interleukin-2 (IL-2) to grow TIL from the fragments. After 3 weeks in TIL-CM the

Pre-Rapid Expansion (pre-REP) TILs were transferred to T-25 flasks for rapid expansion. The REP was performed in upright T-25 flasks by activating 1.3 X 10^5 pre-REP TIL with 26 X 10^6 allogenic, irradiated (5000cGy) PBMC feeder cells with 30ng/ml OKT3 (anti-CD3; Abbott Park, IL) in 1:1 mixture of TIL-CM and AIM -V (Invitorgen). TILs expanded from tumor fragments were harvested after 5 weeks and stained for CD8 expression and recognition of the HLA-A2 MART-1 peptide tetramer. TILs were designated as pre-REP and were re-stimulated with MART-1 peptide. The TILs were expanded for another 12 days and diluted as needed with AIM -V and IL-2 to keep the viable cell density in the range of 1–2 × 10^6/ml. The post-REP TILs were isolated and washed in TIL-CM and rested for 3–6 hrs. before re-stimulation. Expanded TILs were routinely stained for human T cell differentiation markers using fluorochrome-conjugated mAb recognizing CD3, CD4, CD8, CD27, CD28, CD57, and CD62L obtained from BD Biosciences (San Jose, CA) or eBiosciences (La Jolla, CA). TILs were stained with HLA-A2 MART-1 peptide (ELAGIGILTV) tetramer (Beckman Coulter, Fullerton, CA) to track changes in the MART-1–specific $CD8^+$ subpopulation. The stained cells were acquired by using a BD FACScanto II flow cytometry analyzer and FACSDiva software (BD Biosciences). Data were analyzed by FlowJo software (TreeStar, San Carlos, CA).

2.3 MAPK Pathway Inhibitors

BRAF ^{V600E} inhibitor GSK2118436 (Selleckchem), and MEK inhibitors GSK1120212 (Selleckchem), and AZD6244 (Selleckchem), were used to treat melanoma cells. Each MAPK pathway inhibitor was titrated for optimal concentration and incubation time (**Figure 3.1B**). Inhibitors were dissolved in DMSO and stored at -80 in aliquots.

2.4 Antibodies and Flow cytometric analyses

Analysis of surface MHC-I expression on melanoma cell lines was carried out by standard flow cytometry methods. Anti-human HLA-A2-APC (BB7.2) , Anti-human HLA A,B,C-APC (w6/32), Anti-human IFN-g-PE (B27), Anti-human CD8-Pacific Blue (SK1), Alexa Flour 647 (IgG2b) Isotype control antibodies were obtained from the following (Biolegend San Diego, CA); Streptavidin-APC was obtained from (BD Biosciences) and HLA antibody A1,A36 –Biotin conjugated was obtained from (US biological). Transduced melanoma cell lines were cultured with BRAF inhibitor or MEK inhibitors and untreated control (RPMI only) for 3hrs. Cells were seeded at 1.0 X 10^6 cells in a 12 well plate. Each inhibitor was diluted in 10mL of growth media to 100nM each and added to each well to a volume of 2mL and a final concentration of 50nM. After treatment melanoma cells were washed, and stained either with HLA-A2, HLA-A, B, C, or HLA-A1 molecules respectively, and analyzed using FACScantoII flow cytometer (BD Biosciences, Jose CA). For TILs intracellular antigens were stained using the fixation and permeabilization protocol from the BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit with BD GolgiPlug™ (BD biosciences San Jose CA). Stained cells were analyzed using a FACScanto II flow cytometer (BD Biosciences San Jose CA). Data was analyzed using Flowjo analysis software (Treestar Ashland OR).

2.5 T-cell assays

For all T-cell assays MART-1 CD8+ specific TILs obtained from infusion product (>98% MART-1 specific) were used. Transduced WM793 cell lines were pulsed with titrated concentrations of MART-1 peptide, and co-cultured with effector MART-1 TILs at a

1:1 ratio (10,000 tumor: 10,000 effector cells) and incubated at 37°C for 4hrs, following a 3hr incubation with BRAF inhibitor (50nM). Transduced Mel888 cell lines were co-cultured with effector MART-1 TILs at a 1:1 ratio at 37°C for 4hrs, following a 3hr incubation with BRAF inhibitor. TILs were collected and cytokine IFN-γ production was measured by intracellular cytokine staining. TIL were incubated with melanoma cell lines for 4hrs in the presence of GolgiStop (BD Biosciences), washed, fixed, permeabilized, and stained using anti-mouse or anti-human IFN-γ conjugated to APC (BD Biosciences). The cells were incubated for 4 hours because GolgiStop is toxic to cells when in culture past 6 hours. Human T-cells were also stained a fluorescently labeled anti-human CD8 antibody conjugated to Pacific Blue (BD Biosciences). Antigen-specific intracellular IFN-γ production by $CD8^+$ T-cells was then determined by flow cytometric analysis.

2.6 Enzyme-linked immunosorbent assay

ELISA's were performed with WM793 and Mel888 transduced cell lines. Each cell line was seeded into 20,000 cells per well in 96 well plates. BRAF inhibitor (GSK2118436) was used to treat cells at concentration of 50nM per well for 3 hrs. Concurrently cells were also incubated without inhibitor for 3hrs in media as a control. Two hours into treatment WM793 cells were pulsed with MART-1 peptide for 1 hr., after an hour the cells were washed with PBS twice and re-suspended in media. After 3 hours of inhibitor treatment MART-1 specific TILs were added to the media at various tumors to TIL ratios and incubated overnight. After 8hrs of incubation, the media was collected from each well and used to determine the amount of IFN-γ secreted from the TIL using the Ready Set GO! ELISA Human IFN-γ kit (eBiosciences). Plates were read using SpectraMax® M5/M5e

Multimode Plate Reader and program. Data was analyzed with the Excel spreadsheet program.

2.7 Western Blotting

BRAF WT cell lines HS294T and Mewo, BRAF mutant melanoma cells, WM793 and Mel888 were incubated for three hours in BRAF and/or MEK inhibitor at 50nM and 100nM concentrations. Cell lysates were prepared and protein content was normalized using the BCA method (Thermo-Fisher Rockford, IL). Membranes were probed with antibodies against total MEK, P-MEK, total MAPK, P-MAPK (Cell signaling) and total ERK2 (Santa Cruz Biotechnology). Protein was analyzed using the Scientific Pierce Fast Western Blot Kit (Thermo-Fisher Rockford,IL).

2.8 Internalization Assay

Untransduced and HLA-A2 transduced Mel888, WM793 and Mewo cells were incubated in BRAF inhibitor (GSK2118436) for 3 hrs. in 6 well plates at 1.0 X 10^6 cells per well. To determine the effect of the BRAF inhibitor on internalization of MHC-I, drug was washed off the cells with PBS and the cells were re-suspended in drug-free media. Next, each plate was placed into 4 degree at different time points 0 minutes (point directly after drug removal), 30, 60, 90, and 180 minutes respectively to stop internalization. After the final time point was collected the cells were then washed and stained with HLA-A2 APC (Biolegend, San Diego, CA) antibody at 1:200 dilutions. Stained cells were analyzed using a FACScanto II flow cytometer (BD Biosciences San Jose CA). Data was analyzed using Flowjo analysis software (Treestar Ashland OR).

2.9 Confocal Microscopy

All cells were seeded and plated in Ibidi 12-well chamber on microscope glass slides (Ibidi, Verona, WI). For internalization steady state assays all cells were cultured in RMPI 1640 media (GIBCO Grand Island, NY) with 10% FBS (invitogen), 20mM HEPES (Invitrogen). Mel888 and WM793 cells were placed on ice to stop internalization and labeled with AF488 antibody (Biolegend) for 30 minutes. The cells were then incubated for 0, 30, 60, and 180 minute time points. The cells were washed twice with PBS, and fixed with 4% PFA for 10 minutes. At each time point the cells were taken out and fixed, after each sample was fixed the cells were then stained for surface MHC-I using goat anti mouse AF555 antibody (Biolegnd) for 30 minutes on ice. After the surface is stained the nucleus is then labeled with Hoechst for 10 at room temperature, then washed twice. The cells were then imaged using Leica SP2 confocal microscope with 63X oil lens and 1.4 N/A.

To analyze internalization with or with BRAF inhibitor the cells were first labeled at 14 degrees for 30 minutes with MHC-I antibody AF488 (Biolegend). The cells were washed twice with PBS. The cells were then treated with either 50nM of dabrafenib diluted in DMSO (Selleckchem) for 90 minutes or untreated control with only DMSO (invitogen). The time point 90 minutes was used because the cells become very unhealthy with repeated washings and treatment at later time points. After 90 minutes the cells were then washed, and fixed with 4% PFA for 10 minutes, and surface MHC-I was labeled with goat anti mouse AF555 (Biolegnd) at room temperature for 1 hour. After the surface was stained the nucleus was then labeled with Hoechst for 10 at room temperature, and the cells were

washed twice. The cells were then imaged using Leica SP2 confocal microscope with 63X oil lens and 1.4 N/A.

2.10 Statistical analysis

Graph Pad Prism 6 was used for graphing and statistical analysis. A student's t- test was used to analyze the statistical significance of all flow data. A one-way ANOVA test was used to analyze confocal microscopy quantification data. A p-value less than or equal to 0.05 was the cut-off to determine the significance of the statistics.

CHAPTER III:

RESULTS

Overview

MHC-I expression can be upregulated in BRAF mutant melanoma cells following MAPK pathway inhibition [67]. Our experiments sought to shed light on the mechanism behind the modulation of MHC-I expression and the specific role that the MHC-I cytoplasmic tail may play in this phenomenon. It is known that the tyrosine (Y320) encoded by exon 6 and amino acids encoded by exon 7 of the MHC-I cytoplasmic tail are involved in MHC-I trafficking [52,53]. Therefore, we hypothesized that phosphorylation of the tyrosine (Y320) or serine (S355) from exon 7 may be controlled by the oncogenic MAP kinase signaling pathway, thus affecting MHC-I intracellular trafficking. In order to determine this, we examined BRAF mutant melanoma cell lines to further understand the modulation of MHC-I by the oncogenic MAP kinase pathway and to identify a possible mechanism behind regulation of MHC-I.

3.1 Inhibition of the MAP kinase pathway increases HLA-A, B, C surface expression in BRAF mutant cell lines.

In previously published reports, the increase in MHC-I expression after MAP kinase pathway inhibition was observed after 24-72 hours of treatment. However, these late time points pointed to transcriptional upregulation of MHC-I, which was of concern in our study. We therefore wanted to focus on earlier time points, less than 3 hours that may uncover post-transitional modifications as opposed to the effects of transcription factors that can take place within 24hrs of treatment [72].

To determine an optimal time point for drug treatment to inhibit the MAP kinase pathway and to induce changes in MHC-I expression we tested the BRAFi at times 0

minutes, 30 minutes, 60 minutes, and 180 minutes. We then performed flow cytometry analysis to determine the level of total MHC-I on the cell surface at each time point. The results in **Fig.3.1A** show that after 180 minutes of MAPK pathway inhibition, MHC-I is maximally upregulated on the cell surface in comparison to the untreated control.

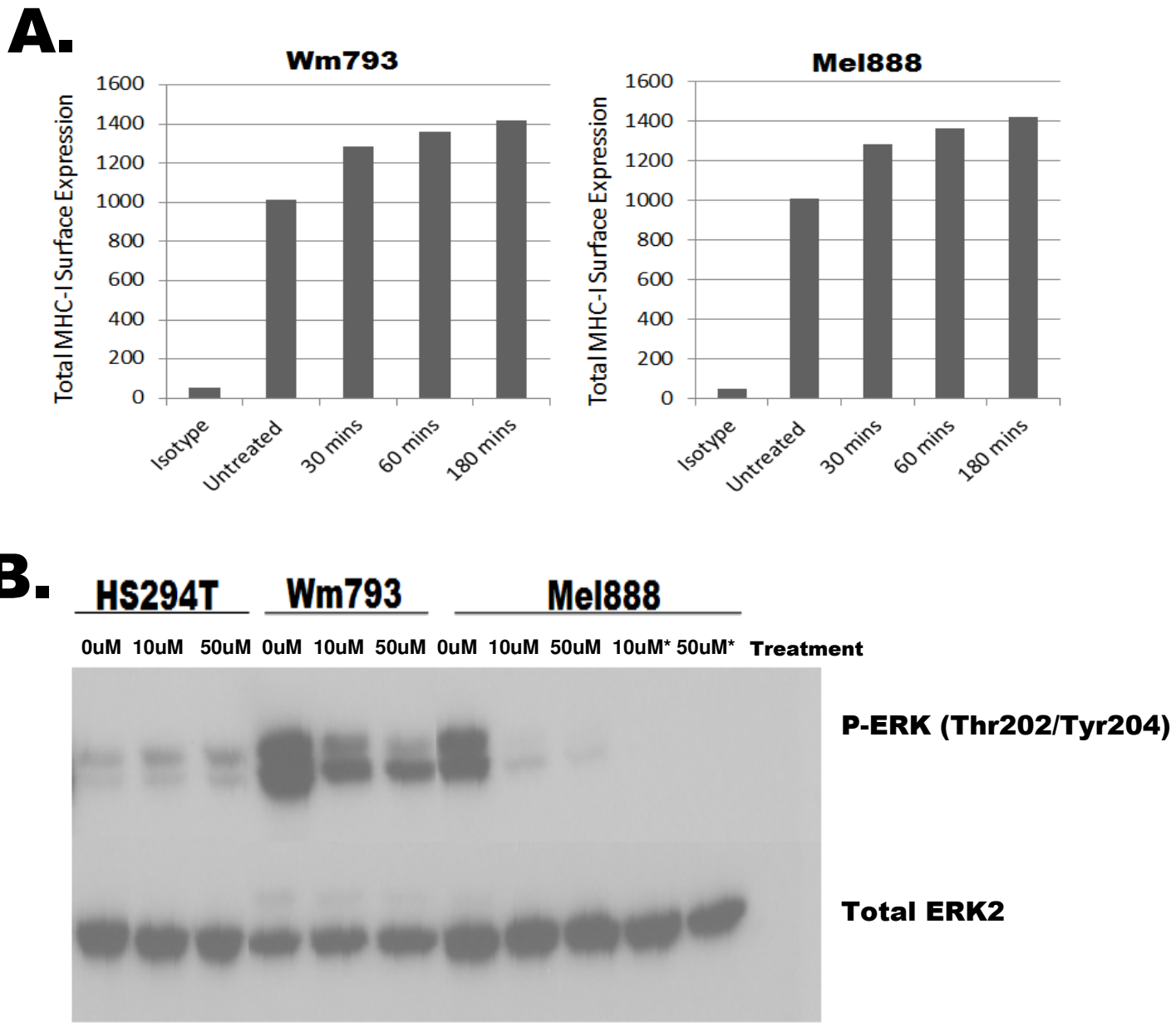
To determine the kinetics of blocking the BRAF signaling pathway, we evaluated the levels of MAPK phosphorylation as readout for MAPK pathway activation. Total ERK was used as a control for phospho-ERK. The results shown in **Fig. 3.1B** reveal that the level of ERK phosphorylation was substantially reduced after 3 hours of treatment using both a BRAF inhibitor (Dabrafenib) and a MEK inhibitor (Trametinab) for 3 hours at low concentrations (10nM), or high concentrations (50nM), compared to vehicle (DMSO) treated cells. In addition, a BRAF WT control cell line, HS294T, showed no change in the level of phospho-ERK following treatment with the BRAF inhibitor (Dabrafenib. These results confirmed that the MAPK signaling pathway was blocked at the drug concentrations and time points tested.

Next we examined the effects of two clinically used MAPK pathway inhibitors, Dabrafenib, and Trametinab on the surface expression of total MHC-I molecules. We treated melanoma cells for 3 hours at a 50nM concentration and as a control, we used DMSO only (vehicle). The levels of MHC-I were observed by flow cytometry and mean fluorescence intensity (MFI) was measured to determine difference in total MHC-I expression in two BRAF mutant cells lines (Mel888 and WM793) and one BRAF WT line (HS294T) (**Fig. 3.1C).** The results show that MHC-I was upregulated in the BRAF mutant cells, while the level of MHC-I did not change in the BRAF WT line. These results ultimately show that the

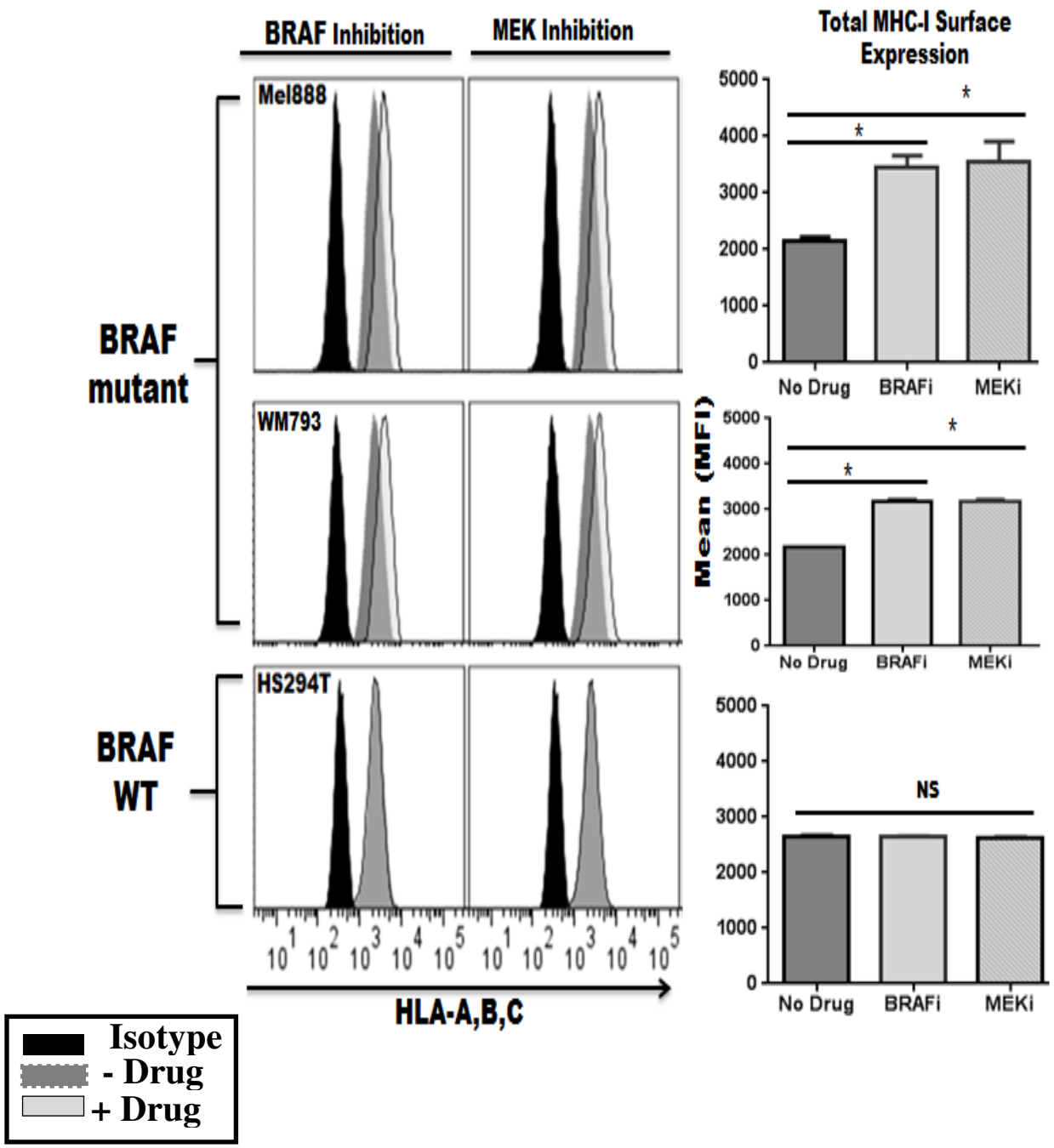
oncogenic MAPK signaling pathway can affect the level of MHC-I surface expression in melanoma cells and that MAPK inhibition can reverse these effects.

Figure 3.1 Inhibition of the MAP kinase pathway increases HLA-A, B, C surface expression in BRAF mutant cell lines. (**A**) Mel888 and WM793 BRAF mutant cell lines were treated with DMSO (vehicle) or dabrafenib for 30 minutes and 3 hours later total surface HLA-A,B,C was stained with W6/32 antibody and cells were analyzed by Flow cytometry. Values represent the mean florescence intensity (MFI) (**B**) Cells were treated with vehicle (DMSO), 10uM, or 50uM of dabrafenib (BRAFi). Mel888 was also treated with Trametinib (MEKi) (indicated by *). Whole cell lysates were prepared and levels of phospho-ERK and total ERK were analyzed by immunoblotting. **(C)** Two BRAF mutant cell lines, Mel888 and WM793 and one BRAF WT cell line, HS294T were treated with DMSO (vehicle), dabrafenib(BRAFi) and trametinib (MEKi) for 3 hours. Following treatment the cells were stained with W6/32 antibody to measure HLA-A, B, C and analyzed by flow cytometry. Left panels show shifts of MHC-I as histograms, and the right panels show actual MFI of MHC-I in a bar graph format. These experiments were repeated at least four times to confirm results. (* indicates $p < 0.05$; NS: not significant).

Figure 3.1


A.
Wm793
Mel888
Total MHC-I Surface Expression
1600
1400
1200
1000
800
600
400
200
0
Isotype
Untreated
30 mins
60 mins
180 mins
B.
HS294T
Wm793
Mel888
0uM 10uM 50uM 0uM 10uM 50uM 0uM 10uM 50uM 10uM* 50uM* Treatment
P-ERK (Thr202/Tyr204)
Total ERK2

c.


BRAF Inhibition
MEK Inhibition
Mel888
WM793
HS294T
BRAF mutant
BRAF WT
HLA-A,B,C
Isotype
- Drug
+ Drug
Total MHC-I Surface Expression
Mean (MFI)
No Drug
BRAFi
MEKi
NS

3.2 Creation of cell lines expressing HLA-A2 cytoplasmic tail variants

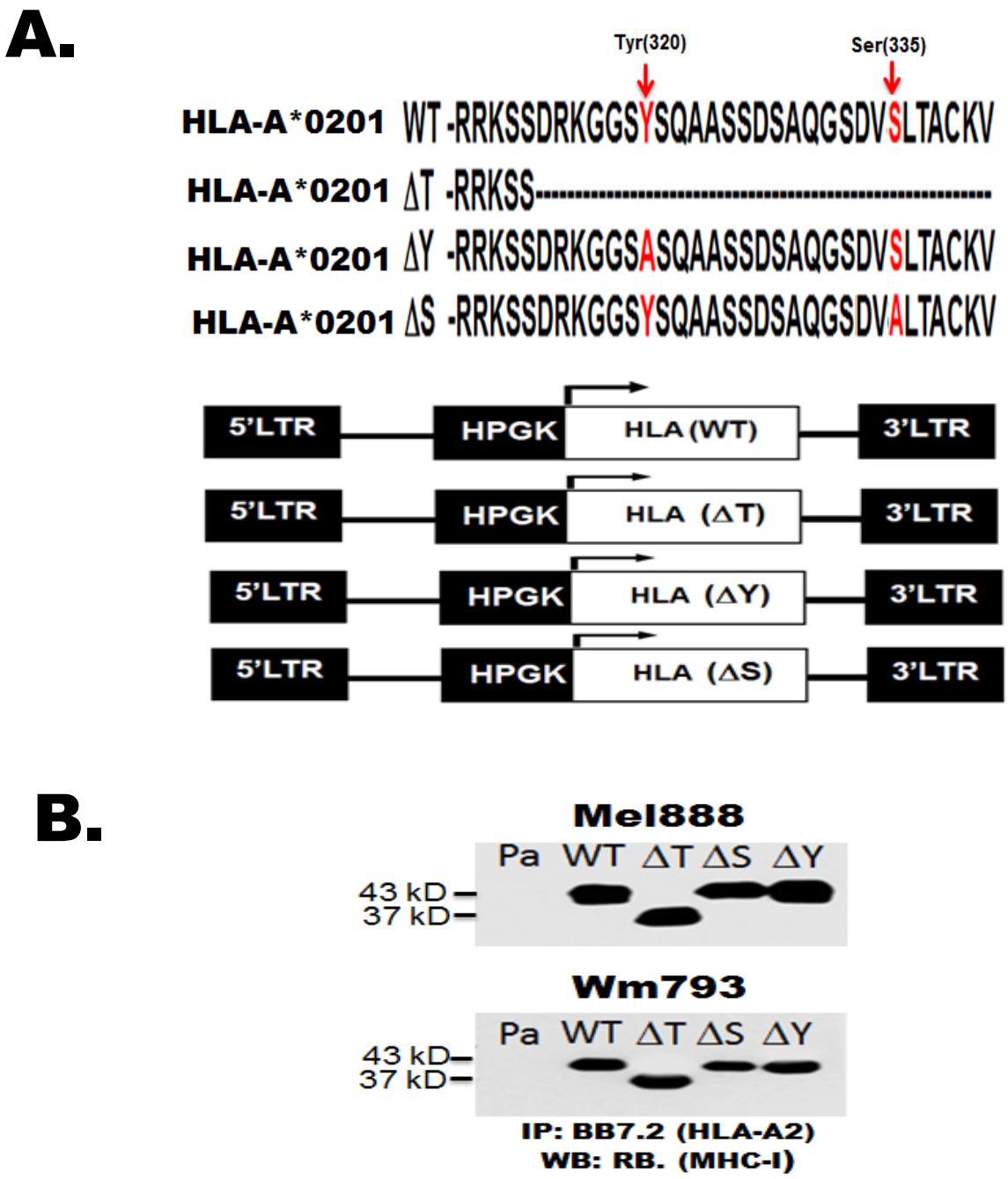
To determine if the MHC-I cytoplasmic tail was involved in the modulation of MHC-I by the MAP kinase pathway, cell lines transduced with cytoplasmic tail mutations of HLA-A2 were made. The melanoma cell lines Mel888 and WM793 were selected for transduction because they lack the expression of endogenous HLA-A2, and also possess a targetable BRAF activating mutation (V600E). Lentiviral vectors [57] were used to transduce the cell lines with WT HLA-A2; ΔT-A2, containing a complete deletion of the cytoplasmic tail; ΔS, a point mutant that replaces the serine to an alanine at S335, and ΔY, containing a similar point substitution to tyrosine(**Fig 3.2 A**). The transduced cells were then sorted, and immunoblotting was used to confirm that the correct HLA-A2 tail variant was present in each cell line (**Fig 3.2 B**). Surface expression of HLA-A2 was checked by flow cytometry to ensure that the surface expression comparable was between all cell lines expressing the HLA-A2 variants (**Fig 3.2 C**).

Following transduction of the BRAF mutant cells, we next wanted to determine if the inhibitors were able to successfully block the MAPK signaling pathway in the HLA-A2 transduced cell lines. We evaluated the levels of MEK and ERK2 phosphorylation as readout for MAPK pathway activation in the WT-A2 and ΔT-A2 lines. Total ERK and MEK were also used as a control. The results in **Fig. 3.2 D** reveal that the levels of MAPK phosphorylation were significantly reduced after 3 hours of treatment with both the BRAF inhibitor (Dabrafenib) and the MEK inhibitor (Trametinab). The cells were treated with a low concentration (10nM), a high concentration (50nM), and a vehicle (DMSO). The results

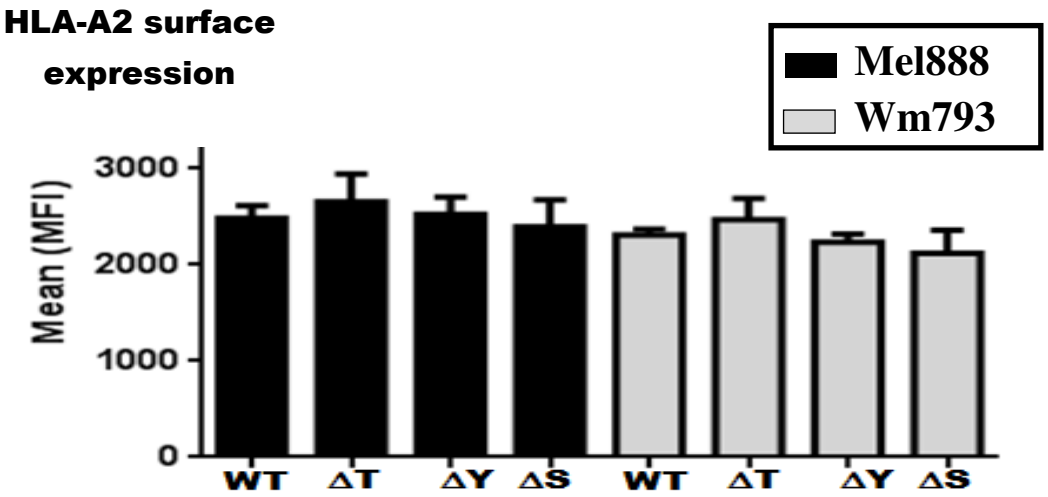
also show that 50nM of drug was sufficient to block ERK and MEK phosphorylation within 3 hours.

Figure 3.2. **Creation of cell lines expressing HLA-A2 cytoplasmic tail variants (A and B**) Amino acid sequences of the cytoplasmic domains of WT, ΔT, ΔS, and ΔY HLA-A*0201. Tyrosine 320 and Serine 335 phosphorylation sites are depicted in red. *Bottom*, Lentiviral vectors used to transduce BRAF mutant melanoma cells. The human phosphoglycerate kinase (hPGK) promoter was used to drive the expression of WT, ΔT, ΔS, and ΔY isoforms of HLA-A*0201. **(C)** Mel888 and WM793 melanoma cells were transduced to express comparable levels of surface HLA-A*0201, as determined by HLA-A2-specific western blot and mAb staining and flow cytometry. (**D**) Cells were treated with vehicle untreated (DMSO), 10uM, or 50uM of dabrafenib (BRAFi) and trametinib (MEKi). Whole cell lysates were prepared and levels of phospho-MAPK and ERK were analyzed by immunoblotting.

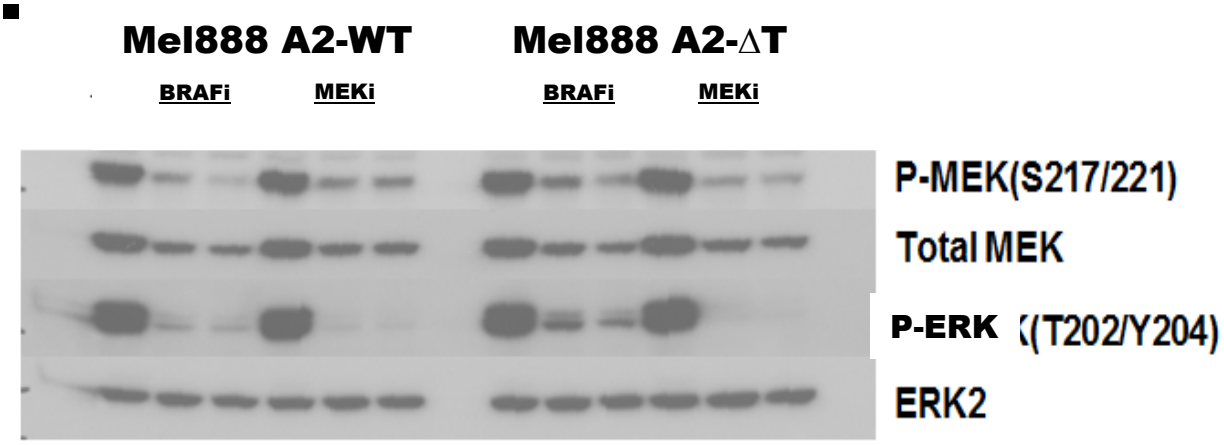
Figure 3.2


A.
Tyr(320)
Ser(335)
HLA-A*0201 WT -RRKSSDRKGGSYSQAASSDSAQGSDVSLTACKV
HLA-A*0201 ΔT -RRKSS----------
HLA-A*0201 ΔY -RRKSSDRKGGSASQAASSDSAQGSDVSLTACKV
HLA-A*0201 ΔS -RRKSSDRKGGSYSQAASSDSAQGSDVALTACKV
5'LTR
HPGK
HLA (WT)
3'LTR
HLA (ΔT)
HLA (ΔY)
HLA (ΔS)
B.
Mel888
Pa WT ΔT ΔS ΔY
43 kD
37 kD
Wm793
IP: BB7.2 (HLA-A2)
WB: RB. (MHC-I)

c.


HLA-A2 surface expression
Mel888
Wm793
Mean (MFI)
3000
2000
1000
0
WT
ΔT
ΔY
ΔS
WT
ΔT
ΔY
ΔS

D.


Mel888 A2-WT
BRAFi
MEKi
Mel888 A2-ΔT
BRAFi
MEKi
P-MEK(S217/221)
Total MEK
P-ERK (T202/Y204)
ERK2

3.3 The MAPK pathway regulates the surface expression of MHC-I in a cytoplasmic tail-dependent matter.

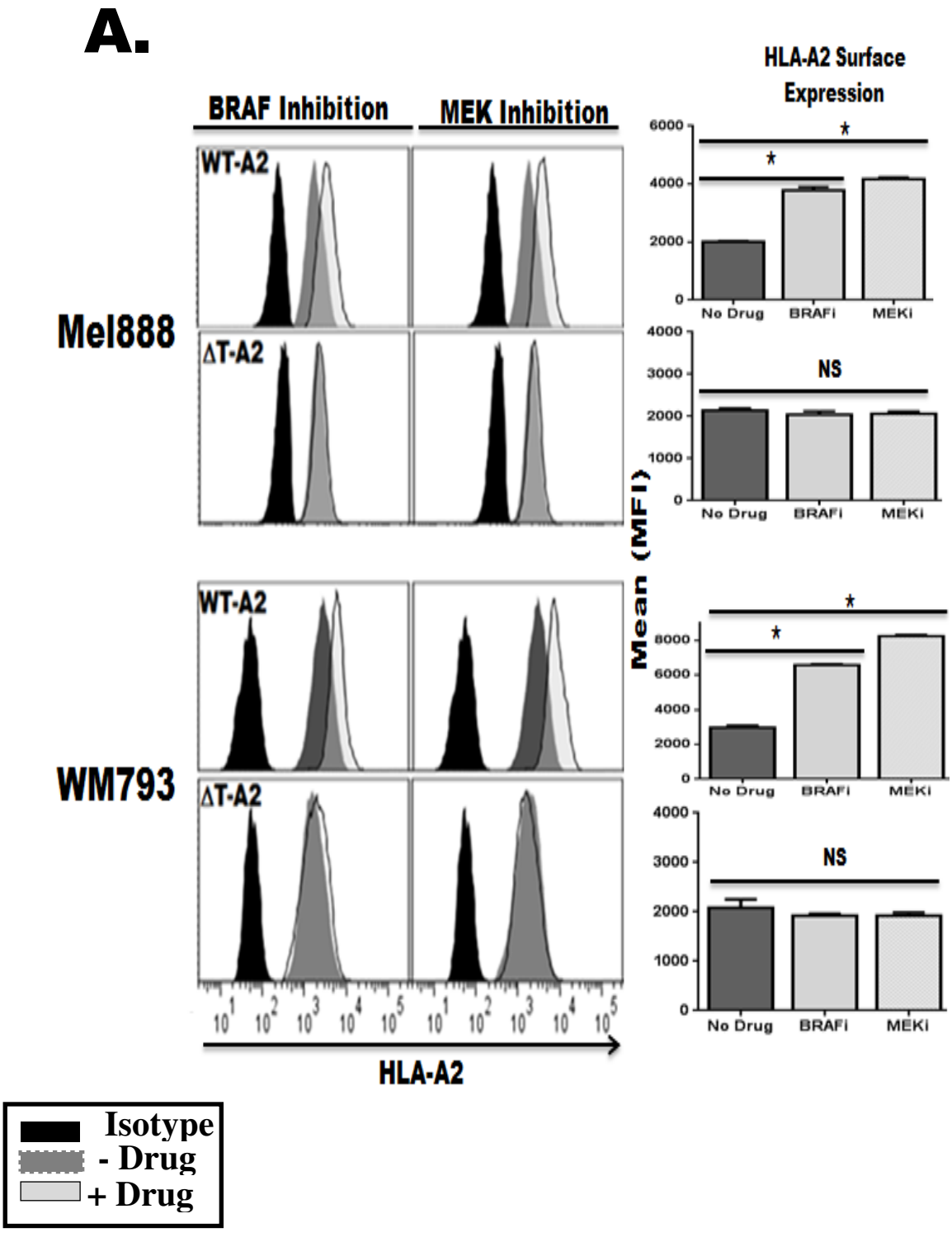
To determine if the MAP kinase pathway modulation of MHC-I was dependent on the cytoplasmic tail, we analyzed HLA-A2 WT and ΔT-A2 transduced BRAF mutant cell lines Mel888 and WM793. Each were treated with DMSO (vehicle), dabrafenib, or trametinib for 3 hours. Following treatment, the cells were stained with HLA-A2 specific antibody (BB7.2) and analyzed by flow cytometry to determine the levels of HLA-A2 on the cell surface following treatment (**Fig. 3.3A**). The results revealed that there was an upregulation in the HLA-A2 transduced WT cell line, while the tailless cell line showed no change in MHC-I surface expression. Therefore, indicating that the MHC-I cytoplasmic tail must be involved the upregulation of MHC-I on the cell surface following BRAF inhibitor treatment.

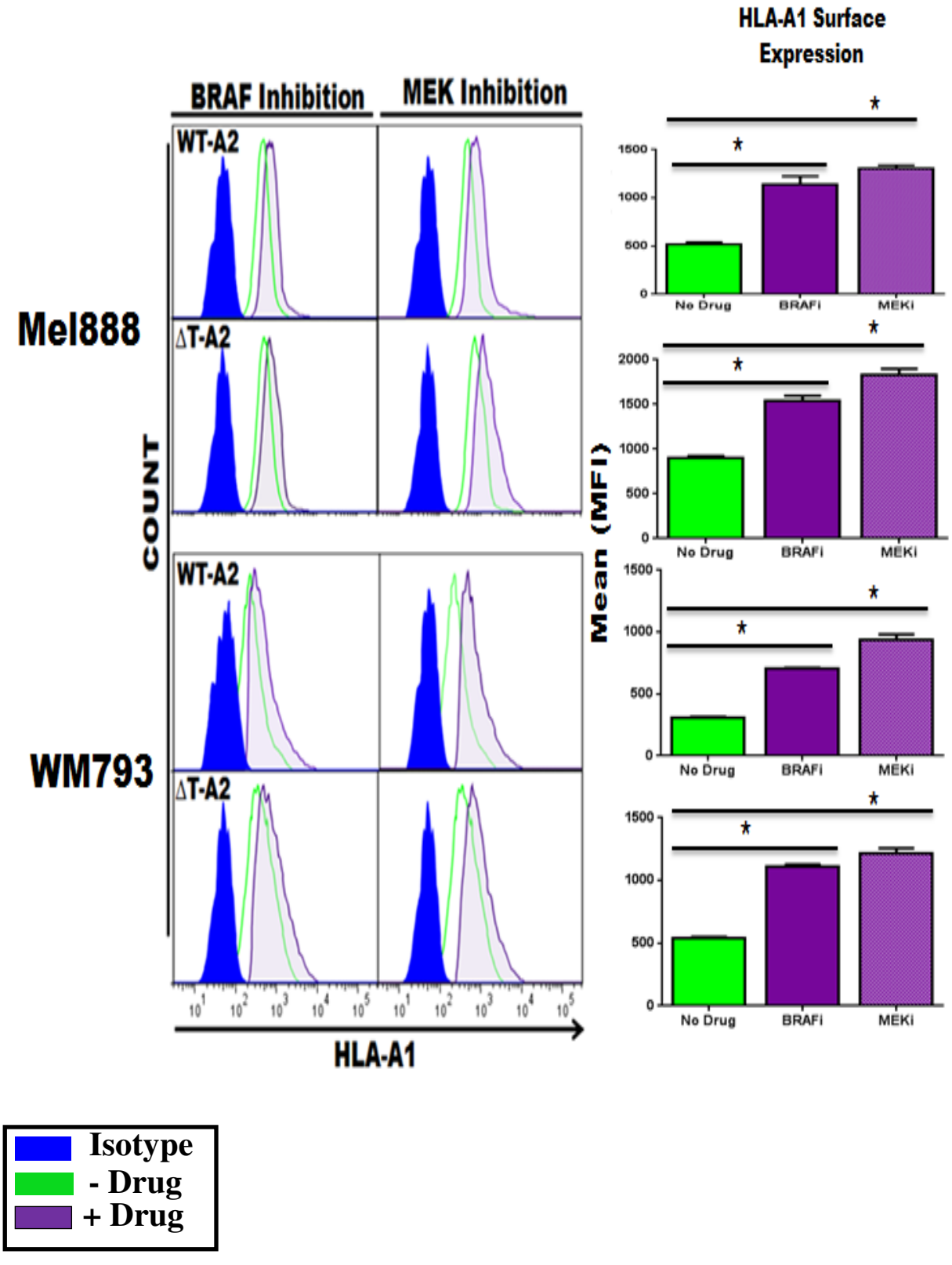
In these experiments, we also analyzed HLA-A1 as an endogenous control in all of our HLA-A2 transduced cell lines. We used HLA-A1 because it has a WT cytoplasmic tail and should there be subjected to surface expression changes after inhibitor treatment. We examined the WT and tailless HLA-A2 cell lines; each were treated with 50nM of dabrafenib or trametinib for 3 hours, and following treatment the cells were stained with an HLA-A1 specific antibody to observe the surface expression level post treatment of drug. As expected, the results revealed that HLA-A1 increased in both the WT-A2 and tailless HLA-A2 cell lines, similar to the total MHC-I increase shown in **Fig. 3.3B**.

Figure 3.3 The MAPK pathway regulates the surface expression of MHC-I in a cytoplasmic tail dependent matter.

(**A**) HLA-A2 transduced Mel888 and WM793 cells were treated with DMSO (vehicle), dabrafenib (BRAFi), or trametinib (MEKi) for 3 hours. Following treatment, the cells were stained with HLA-A2 antibody (BB7.2) and analyzed by flow cytometry. Left panels show MHC-I surface expression as histograms and the right panels show MFI of MHC-I as a bar graph. (**B**) HLA-A1 was measured by flow cytometry in Mel888 and WM793 cells transduced with WT-A2 and ΔT-A2 following treatment with 50uM BRAF or MEK inhibitor for 3 hours. Left panels show changes of MHC-I surface expression as histograms and the right panels show MFI of MHC-I in a bar graph format. These experiments were repeated at least four times with similar results. (* indicates $p < 0.05$; NS: not significant).

Figure 3.3


A.
BRAF Inhibition
MEK Inhibition
HLA-A2 Surface Expression
Mel888
WM793
WT-A2
ΔT-A2
Mean (MFI)
No Drug
BRAFi
MEKi
NS
HLA-A2
Isotype
- Drug
+ Drug


HLA-A1 Surface Expression
BRAF Inhibition
MEK Inhibition
Mel888
WM793
WT-A2
ΔT-A2
COUNT
HLA-A1
Mean (MFI)
No Drug
BRAFi
MEKi
Isotype
- Drug
+ Drug

3.4 Highly conserved serine-335 of the MHC-I cytoplasmic tail mediates modulation of MHC-I surface expression by the MAPK pathway.

Since our results revealed a connection between the MHC-I cytoplasmic tail and MAP kinase pathway inhibition, we next wanted to determine which regions of the MHC-I tail might mediate this connection. To determine which part of the MHC-I cytoplasmic tail modulated MHC-I expression, we next analyzed HLA-A2 cytoplasmic tail point mutants ΔY and ΔS. Point mutants of the tyrosine (320) and Serine (S335) were used because they are both putative sites of phosphorylation on the cytoplasmic tail. If the tyrosine or serine are key sites involved in MHC-I modulation, we might be able to see that mutating these sites will abrogate modulation of surface expression of HLA-A2 in the untreated vs the drug-treated cell lines. To determine if the MAP kinase pathway modulation of MHC-I was dependent upon the two conserved phosphorylation sites, we assessed ΔY and ΔS HLA-A2 expression in transduced BRAF mutant cell lines Mel888 and WM793. Each cell line was treated with DMSO (vehicle), dabrafenib, or trametinib for 3 hours. Following treatment, the cells were stained with HLA-A2 specific antibody (BB7.2) and analyzed by flow cytometry to determine the levels of HLA-A2 on the cell surface following treatment (**Fig. 3.4A**).

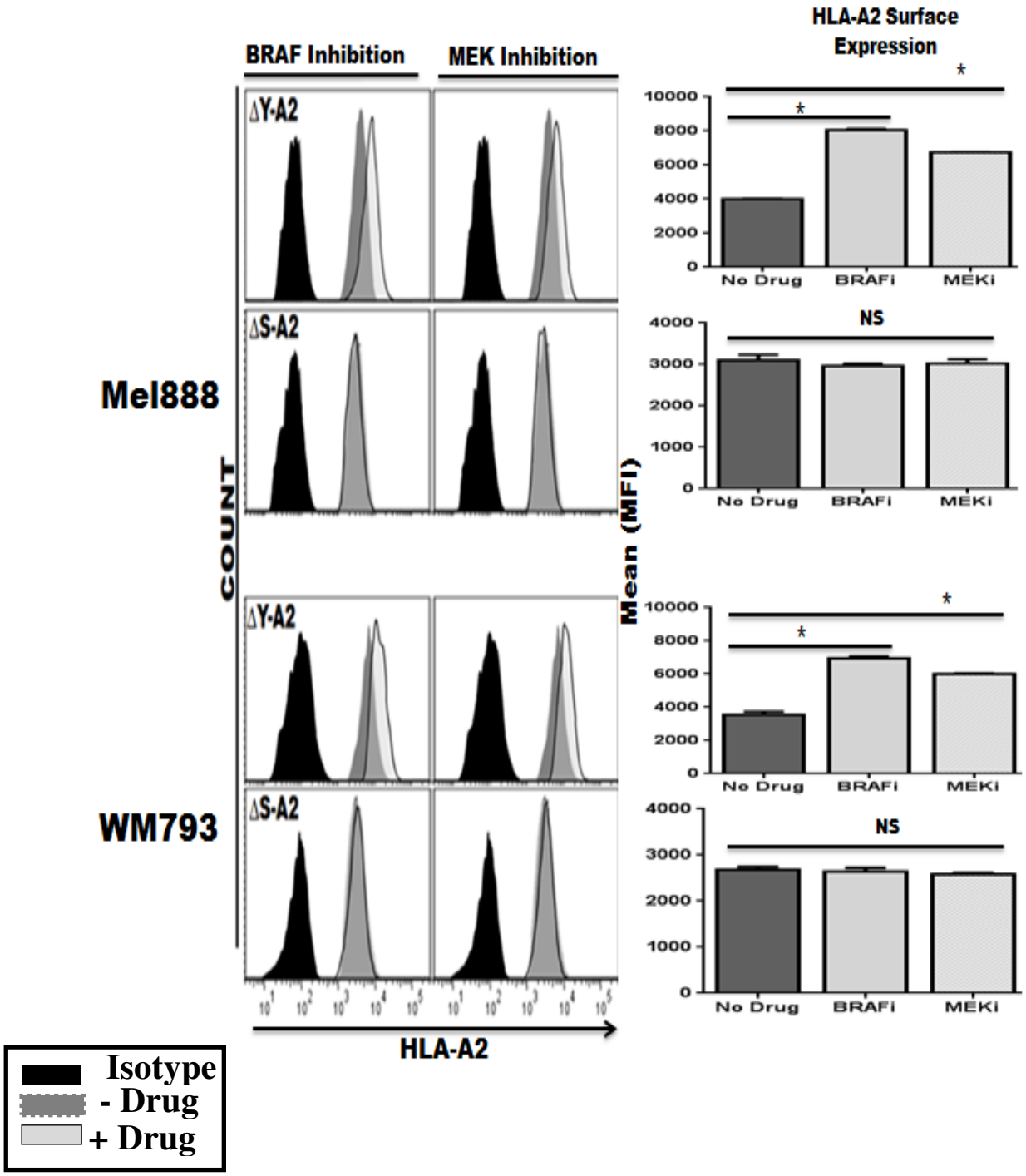
In a similar manner to the previous experiment, we analyzed at endogenous HLA-A1 as a control in the ΔY and ΔS cell lines. Each was treated with 50nM of dabrafenib and trametinib for 3 hours; following treatment, the cells were stained with an HLA-A1 specific antibody to observe the surface expression level post treatment. The results revealed that HLA-A1 expression increased in both the cell lines similar to the total WT MHC-I as expected **(Fig. 3.4B)**.

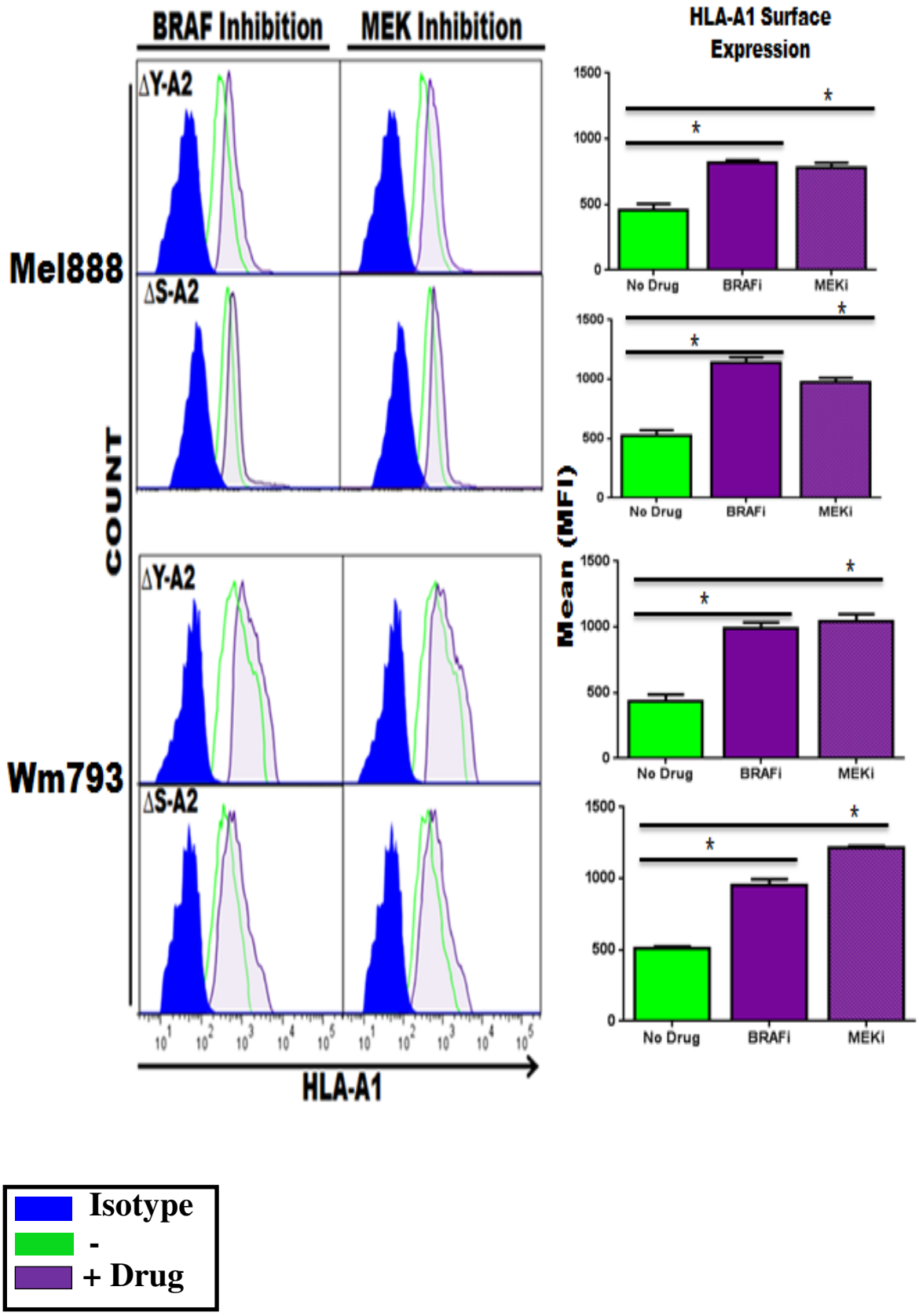
The results revealed that the ΔY-A2 molecule behaved in a similar matter to the WT-A2 molecule, while ΔS-A2 acted similarly to ΔT-A2. These results suggested that the MHC-I cytoplasmic tail modulation is serine-dependent and not tyrosine-dependent. These results are the first to not only connect the cytoplasmic tail to MAP kinase signaling pathway but also to implicate a particular conserved phosphorylation site to the surface expression of MHC-I in tumor cells.

Figure 3.4 Highly conserved serine-335 of the MHC-I cytoplasmic tail mediates modulation of MHC-I surface expression by the MAPK pathway.

(A) Two BRAF mutant cell lines, Mel888 and WM793 were transduced to express HLA-A2 with point mutations to Y320 (ΔY-A2) or S335 (ΔS-A2). Each cell line was treated with DMSO (vehicle), dabrafenib, or trametinib for 3 hours. Following treatment, the cells were stained with BB7.2 HLA-A2 antibody and analyzed by flow cytometry. Left panels show surface expression of MHC-I depicted as histograms and the right panels show MFI of MHC-I expression in a bar graph format. **(B)** HLA-A1 was measured by flow cytometry in two BRAF mutant cell transduced with ΔY-A2 or ΔS-A2 following treatment with 50uM BRAF or MEK inhibitor for 3 hours. Left panels show MHC-I expression as histograms and the right panels show actual MFI of MHC-I as a bar graph. These experiments were repeated at least four times with similar results. (* indicates $p < 0.05$; NS: not significant).

Figure 3.4


BRAF Inhibition
MEK Inhibition
HLA-A2 Surface Expression
ΔY-A2
ΔS-A2
Mel888
WM793
COUNT
HLA-A2
Mean (MFI)
No Drug
BRAFi
MEKi
NS
Isotype
- Drug
+ Drug


BRAF Inhibition
MEK Inhibition
HLA-A1 Surface Expression
Mel888
Wm793
ΔY-A2
ΔS-A2
COUNT
HLA-A1
Mean (MFI)
No Drug
BRAFi
MEKi
Isotype
-
+ Drug

3.5 BRAF inhibitor treatment of BRAF mutant cell lines increases subsequent T-cell recognition and IFN-γ secretion.

To assess the significance of inhibitor-mediated MHC-I upregulation, we next measured the effect of these changes on T cell recognition and activation. Thus, we sought to determine the T-cell response to each transduced HLA-A2 cell line following BRAF treatment. In the first set of experiments, we analyzed $CD8^+$ MART-1 specific TIL (> 90% tetramer recognition) of Mel888 cells which naturally express MART-1 [73]. WM793 cells pulsed with MART-1 peptide were used as targets because they do not express MART-1 protein naturally. Mel888 cells transduced with HLA-A2 variants were treated with the BRAF inhibitor dabrafenib for 3 hours, the cells were washed, and T-cells were added at a 1:1 ratio in triplicate. Golgi block was added to the media to block the secretion of IFN-γ and the cells were co-incubated with T-cells for 8 hours. T-cells were collected and washed, then stained with antibody for $CD8^+$ T-cell markers. T-cells were then permeabilized, and stained for intracellular IFN-γ. All cells were then observed by flow cytometry, with appropriate controls used to detect background levels of IFN-γ (**Fig 3.5A**). TIL alone with no melanoma cells, as well as melanoma cells done with no TIL were used as controls. The results revealed that the TIL co-cultured with WT-A2 and ΔY-A2 HLA-A2 transduced cells produced more IFN-γ after BRAF inhibitor treatment. However, TIL co-cultured with ΔT-A2 and ΔS-A2 transduced cell lines showed no significant increases in IFN-γ secretion consistent with the flow cytometry results. These results show that the inhibition of oncogenic BRAF can have a direct effect on TIL IFN-γ production.

We also repeated the experiment using HLA-A2 transduced WM793 cells, except the cells were incubated with 50nM of MART-1 peptide for the final hour of the 3 hour BRAFi treatment. Similar to the results with Mel888 cells, the results from these experiments correlated well with our MHC-I flow cytometry results. As expected, the T-cells produced more IFN-γ in response to exposure to the cell lines transduced with WT-A2 and ΔY-A2. By contrast, notable were the results of the ΔT-A2 and ΔS-A2 cell lines, which elicited only slight decreases or no change in the frequency of IFN-γ positive CD8 cells in comparison to those cells co-cultured with untreated cells (**Fig 3.5B**).

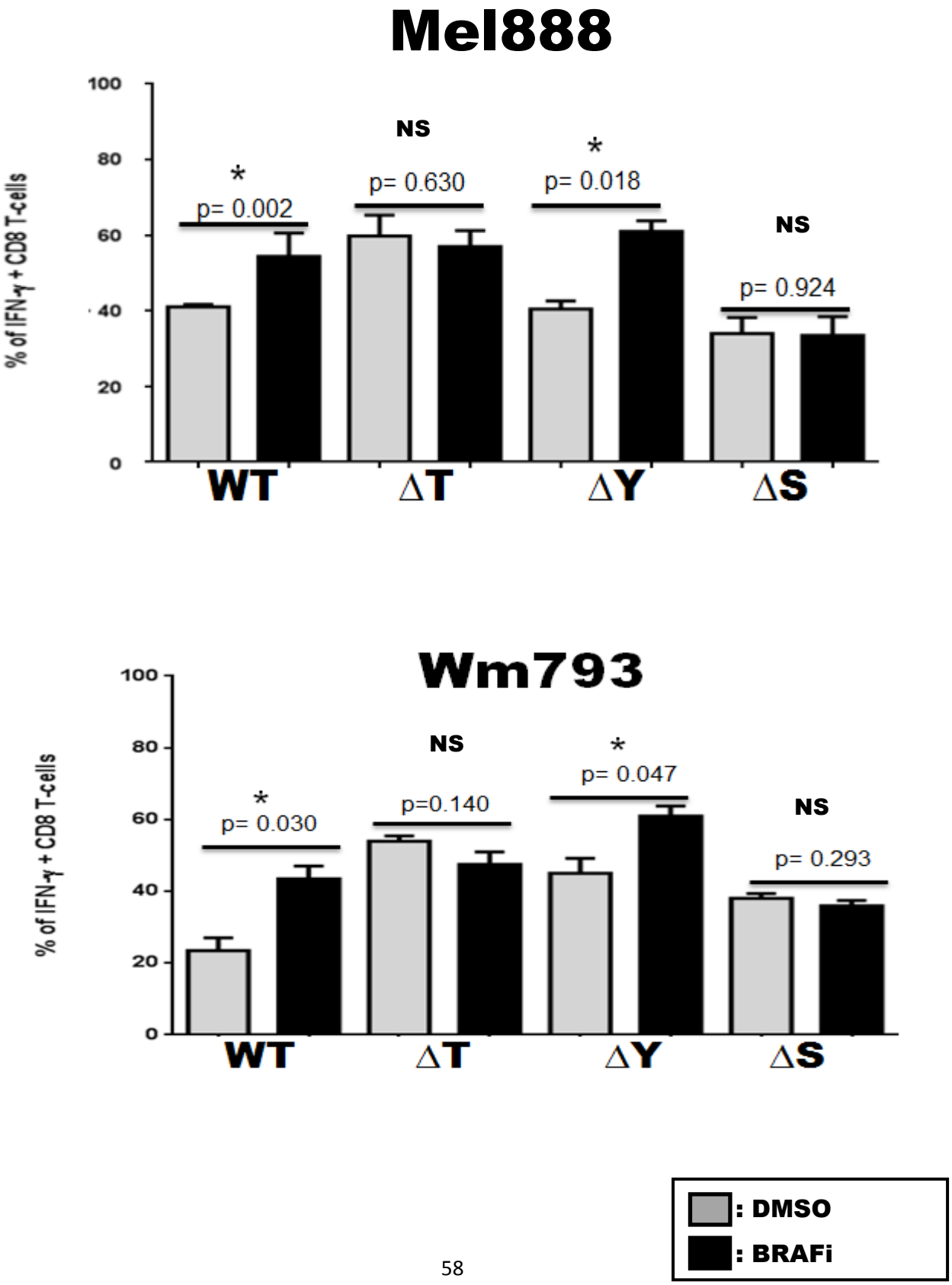
In order to understand how cytokine secretion by the MART-1 T-cells related to antigen presentation, T2 cells were pulsed with increasing concentrations of MART-1 peptide and assessed IFN-γ secretion elicited by T-cells was measured. In parallel, the same MART-1 specific TILs were incubated with Mel888 cells before and after BRAF inhibitor treatment. An IFN-γ ELISA was performed with the transduced Mel888 cells after a 3 hour treatment with BRAFi or with DMSO; each cell line was co-cultured with MART-1 TIL for 8 hours, and we measured the levels of IFN-γ secreted into the supernatant. The results in **Figure 3.5C** show the level of IFN-γ released by the TIL incubated with transduced Mel888 cells in comparison to the level of IFN-γ released in response to peptide-pulsed T2 cells. The top panel shows that the WT-A2 cell line had what would be an equivalent of a 4-fold increase of peptide on the cell surface, following BRAF treatment in comparison to the T2 peptide-pulsed cells. The ΔY-A2 cell line displayed what would equate to a 6-fold increase of peptide on the cell surface when compared to T2 cells pulsed with peptide. These results

demonstrate how the upregulation of MHC-I surface expression has on the cell surface can affect T-cell recognition.

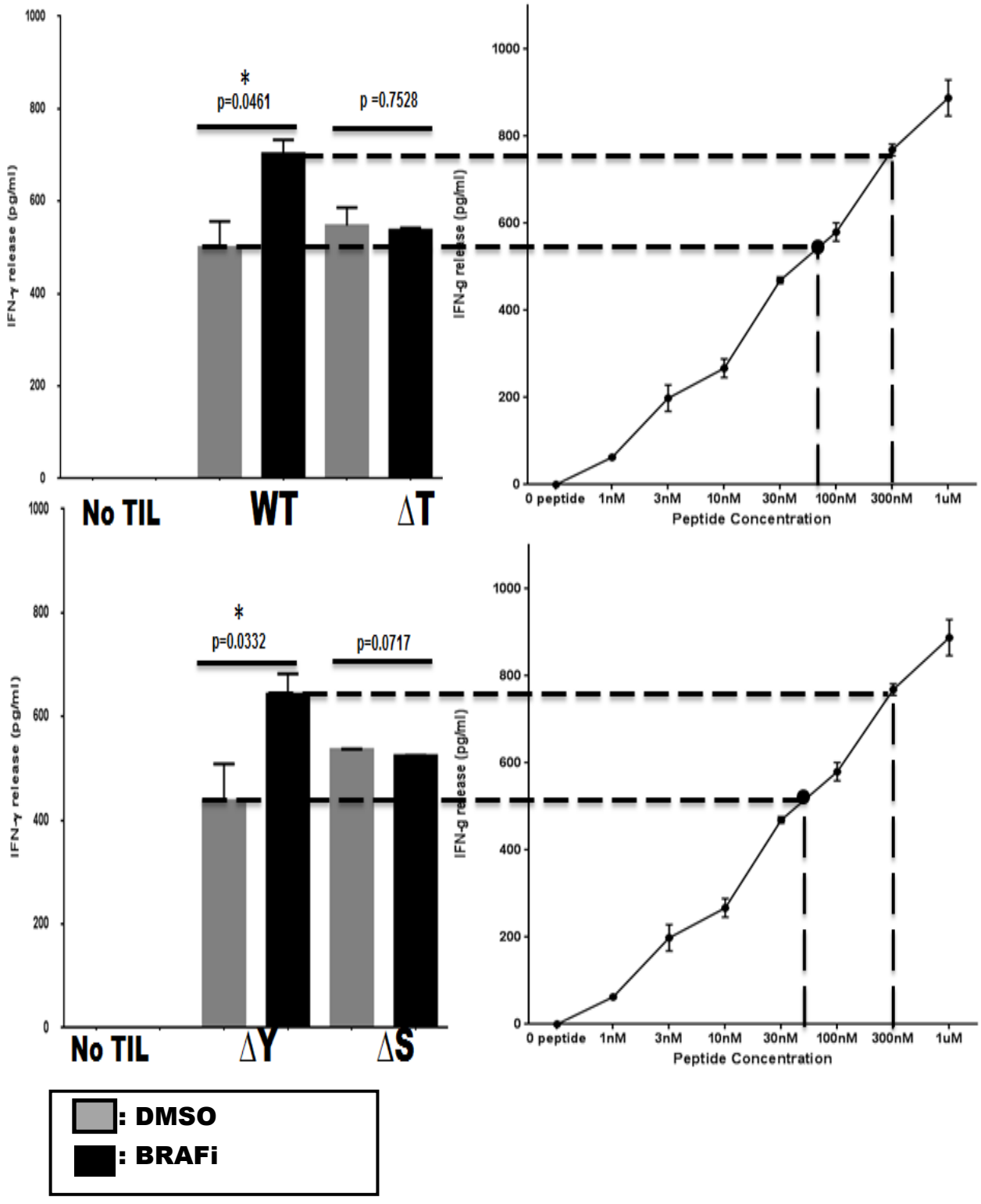
We also measured the level of IFN-γ secreted into the supernatant by MART-1 specific TILS co-cultured with WM793 cells pulsed with 50nM of MART-1 peptide. (**Fig 3.5D**). The results were consistent with our previous experiment: MART-1 TILs co-cultured with WT-A2 and ΔY-A2 expressing cells showed increases of IFN-γ; following BRAFi treatment. By contrast, TILs co-cultured with ΔT-A2 and ΔS-A2 showed no change in IFN-γ released. These results confirm that the MHC-I upregulation by the MAPK pathway plays a potentially significant role in immunological recognition of tumor cells by T-cells. Our results also point to serine-335 as the site that could be the key to this mechanism.

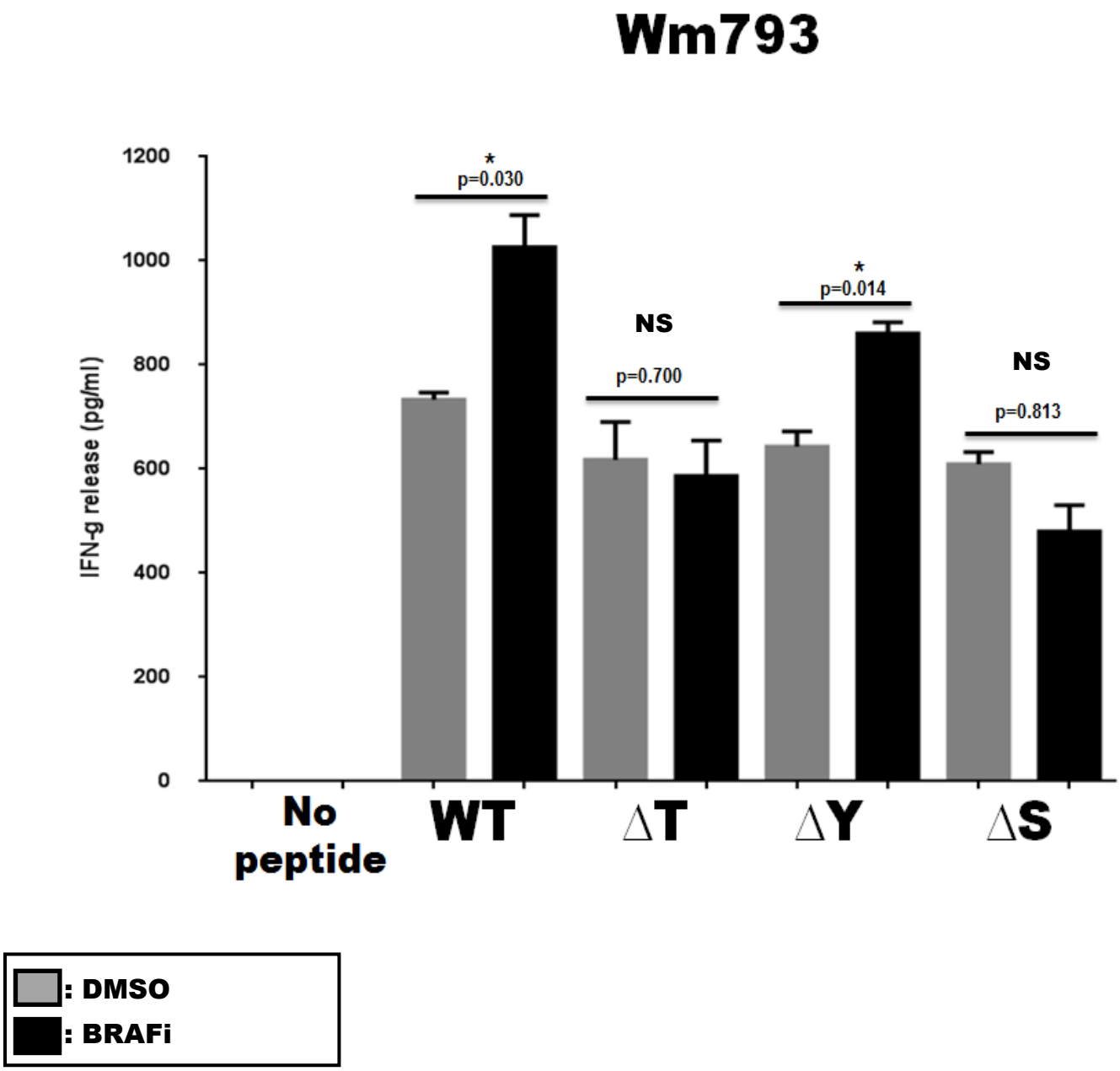
Figure 3.5 BRAF inhibitor treatment of BRAF mutant cell lines increases subsequent T-cell recognition and IFN-γ secretion. **(A)**Frequency of IFN-γ expressing $CD8^+$ T-cells following co-culture with MART-1 expressing Mel888 cells transduced to express WT HLA-A2 or cytoplasmic tail variants of HLA-A2: ΔT, ΔY, and ΔS. Cells were treated with BRAF inhibitor for 3 hours, washed, and then co-cultured for 8 hours with MART-1 specific T-cells. Frequency of IFN-γ positive T-cells are shown for each cell line with or without inhibitor treatment. **(B)** Frequency of IFN-γ cytokine-expressing $CD8^+$ T-cells following co-culture with WM793 cells pulsed with 50nM of MART-1 peptide. Cells were treated with BRAF inhibitor for 3 hours including MART-1 peptide for the final hour, washed, and then co-cultured for 8 hours with T-cells. **(C)** Results of ELISA assay measuring IFN-γ released into the supernatant by MART-1 specific T-cells after 8 hour co-culture with HLA-A2 transduced Mel888 cells, treated with or without BRAF inhibitor for 3 hours. In parallel, T2-cells pulsed with a titrated amounts of MART-1 peptide were assessed as stimulator cells for the same CD8+ T-cells.**(D)** MART-1 peptide pulsed HLA-A2 transduced WM793 cells treated with BRAF inhibitor or DMSO (vehicle) for 3 hours. All experiments were repeated at least three times with similar results. (* indicates $p < 0.05$; NS: not significant).

Figure 3.5


Mel888
% of IFN-γ + CD8 T-cells
100
80
60
40
20
0
*
p= 0.002
NS
p= 0.630
*
p= 0.018
NS
p= 0.924
WT
ΔT
ΔY
ΔS
Wm793
% of IFN-γ + CD8 T-cells
100
80
60
40
20
0
*
p= 0.030
NS
p=0.140
*
p= 0.047
NS
p= 0.293
WT
ΔT
ΔY
ΔS
: DMSO
: BRAFi

Mel888


IFN-γ release (pg/ml)
1000
800
600
400
200
0
*
p=0.0461
p =0.7528
No TIL
WT
ΔT
IFN-g release (pg/ml)
0 peptide
1nM
3nM
10nM
30nM
100nM
300nM
1uM
Peptide Concentration
p=0.0332
p=0.0717
No TIL
ΔY
ΔS
: DMSO
: BRAFi


Wm793
IFN-g release (pg/ml)
1200
1000
800
600
400
200
0
*
p=0.030
NS
p=0.700
*
p=0.014
NS
p=0.813
No peptide
WT
ΔT
ΔY
ΔS
: DMSO
: BRAFi

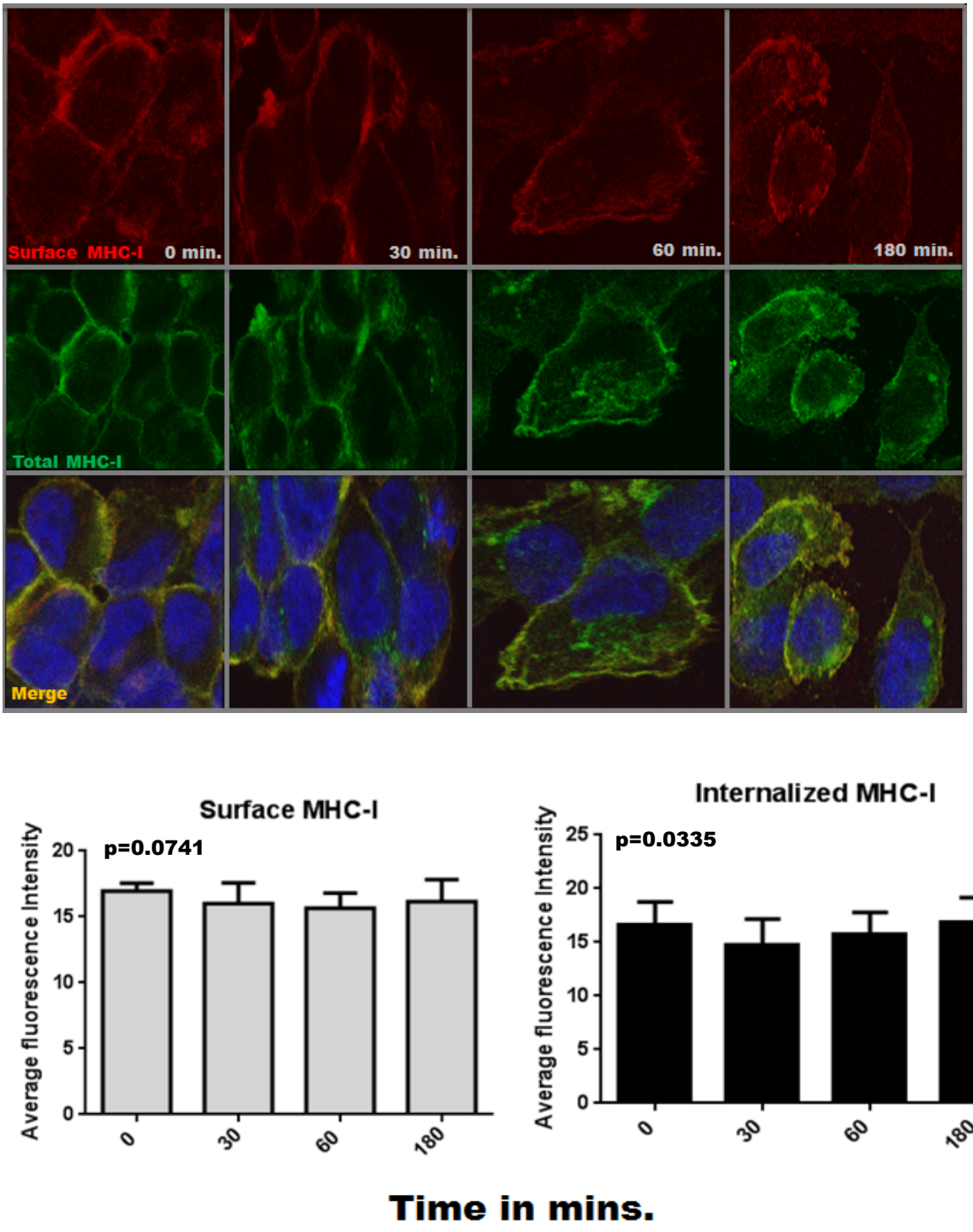
3.6 Assessing the steady-state rate of internalization of MHC-I in BRAF WT and BRAF mutant cell lines.

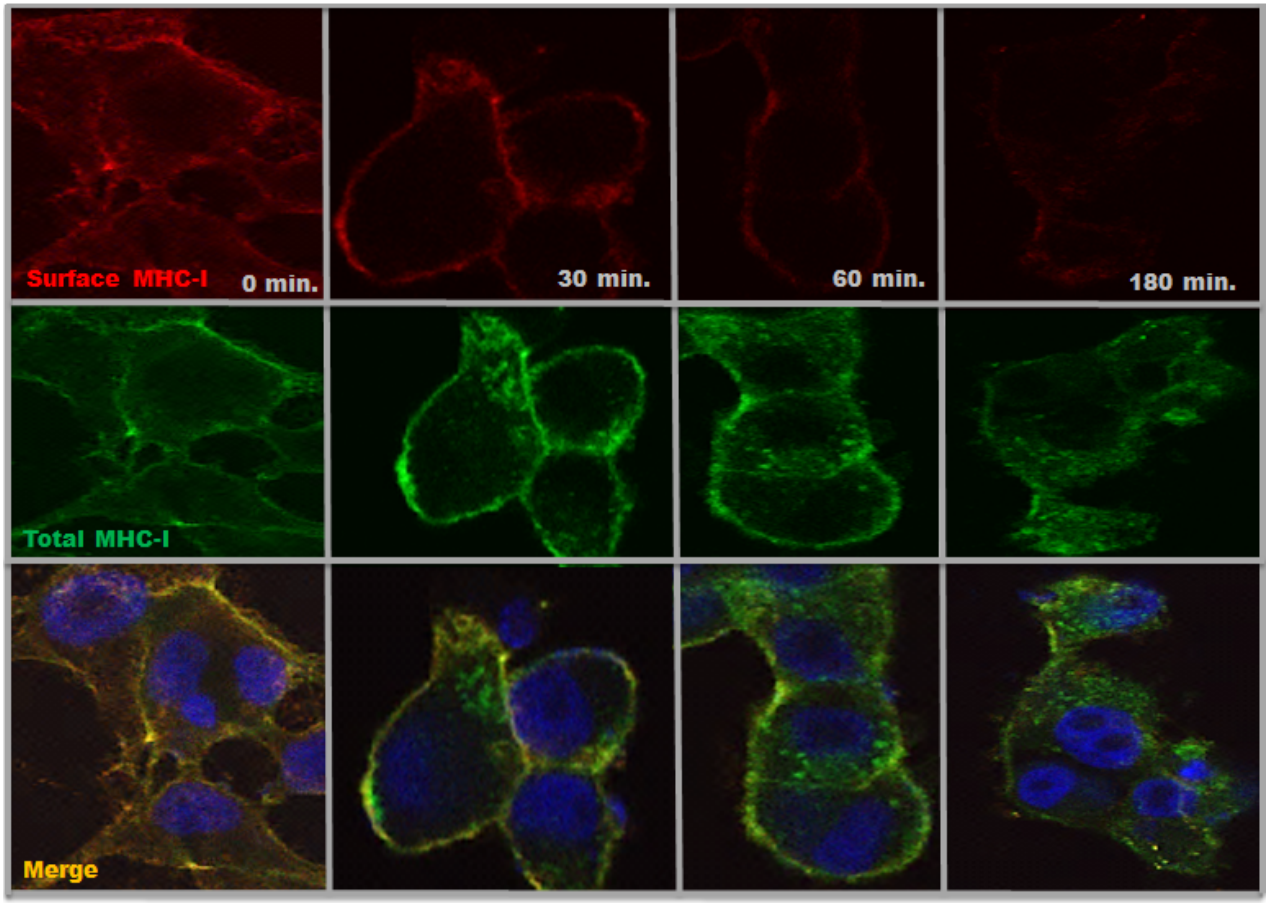
In order to better understand the mechanism behind the oncogenic regulation of MHC-I surface expression; we next initiated a series of confocal microscopy experiments. We first compared the steady state internalization rates of MHC-I in the BRAF mutant cell line Mel888 and the BRAF WT cell line HS294T. Cells were first seeded and plated onto Ibidi 12-well chambers on microscope glass slides. Cells were put on ice to stop internalization, and stained with AF488–labeled with W6/32 antibody to stain for total MHC-I. Cells were incubated at 37 degrees for various time points, after which they were taken out, fixed, and surface stained using goat anti mouse antibody conjugated with AF555. Cells were then observed by confocal microscopy.

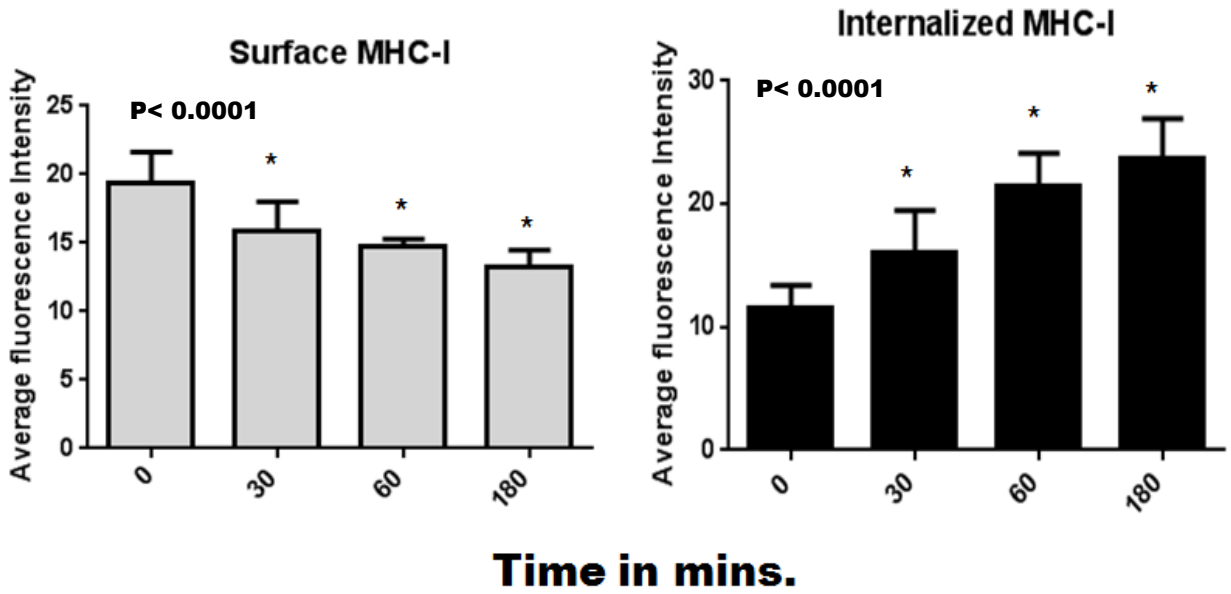
The result for the HS294T cells shows that MHC-I surface expression does not significantly change from baseline to 180 minutes. In addition the level of MHC-I internalized also remains close to baseline levels after 180 minutes (**Fig 3.6A**). By contrast, Mel888 cells showed significant internalization of MHC-I over the same time period (**Fig. 3.6B**). These results reveal that there is a more rapid constitutive rate of MHC-I internalization in Mel888 cells compared to HS294T cells. These results are consistent with our flow cytometry studies (**Fig 3.1C)** and support the notion that oncogenic BRAF is driving the enhanced internalization observed.

Figure 3.6 The steady-state rate of internalization of MHC-I in BRAF WT and BRAF mutant cell lines. (A) Confocal microscopy analysis of the BRAF WT cell line HS294T. The red fluorescence represents surface MHC-I, green is total MHC-I, and the bottom panel shows a merged image (yellow). **(B)** Quantification of the average pixel fluorescence per cell of surface MHC-I (red) and internalized MHC-I (green in merged image). **(C)** Confocal microscopy analysis of the BRAF mutant cell line Mel888. **(D)** Quantification of the average pixel fluorescence per cell of surface MHC-I (yellow in merged image) and internalized MHC-I (green in merged image). All experiments were repeated twice with similar results. *Imaging done by Dr. Zeming Chen*

Figure 3.6


Surface MHC-I
0 min.
30 min.
60 min.
180 min.
Total MHC-I
Merge
Surface MHC-I
p=0.0741
Average fluorescence Intensity
0
5
10
15
20
0
30
60
180
Internalized MHC-I
p=0.0335
Average fluorescence Intensity
0
5
10
15
20
25
0
30
60
180
Time in mins.


Surface MHC-I
0 min.
30 min.
60 min.
180 min.
Total MHC-I
Merge


Surface MHC-I
P< 0.0001
Average fluorescence Intensity
25
20
15
10
5
0
0
30
60
180
Internalized MHC-I
P< 0.0001
Average fluorescence Intensity
30
20
10
0
0
30
60
180
Time in mins.

3.7 BRAF inhibitor slows constitutive MHC-I internalization in a cytoplasmic tail dependent matter.

After determining that MHC-I is rapidly internalized in BRAF mutant cell lines, we next wanted to know if MAP kinase pathway inhibition affected this internalization. Abrogation of MHC-I internalization may lead to the increase in cell surface MHC-I that we have consistently observed after BRAFi treatment. If this is the mechanism, we would expect that following removal of the BRAFi, MHC-I should begin to internalize again and return to baseline surface levels. To test this prediction, we treated Mel888 cells with BRAF inhibitor for 3 hours and measured total MHC-I at different time points, following drug removal. As expected, MHC-I surface expression increased by flow cytometry following inhibitor BRAF treatment in Mel888 cells but not WT BRAF-expressing MeWo cells (**Fig 3.7A**). However, following drug removal, MHC-I surface levels in Mel888 cells progressively decreased until falling back to baseline levels by 3 hours. These results support a model in which BRAF inhibitor treatment halts or slows MHC-I internalization in BRAF mutant cells, which leads to an increase in MHC-I on the cell surface. Since the WT BRAF-expressing cell line, MeWo showed no change in MHC-I following BRAFi treatment and after removal of drug, this supports the idea that oncogenic BRAF is driving the rapid and constitutive internalization of MHC-I.

We next wanted to determine how the MHC-I cytoplasmic tail affected MHC-I internalization in the Mel888 cell line. A similar experiment to the one described above was performed, except that we monitored HLA-A2 surface expression in Mel888 cells transduced with WT-A2 and tailless A2. WT HLA-A2 demonstrated similar dynamics of surface expression changes as total MHC-I in response to BRAFi addition and removal **(Fig**

3.7B). However, tailless HLA-A2 showed no change in MHC-I surface expression following drug addition and removal. These results show that constitutive internalization of MHC-I in Mel888 is not only dependent upon oncogenic BRAF, but also the MHC-I cytoplasmic tail.

Figure 3.7 BRAF inhibitor slows constitutive MHC-I internalization in BRAF mutant melanoma cell line. **(A)** Dynamics of total MHC-I surface expression in Mel888 cells following addition and removal of the BRAF inhibitor. BRAF-WT expressing MeWo cells were used as a negative control. Cells were observed by flow cytometry at different time points, following staining with W6/32 mAb. **(B)** Dynamics of HLA-A2 surface expression following addition and removal of BRAF inhibitor in Mel888 cells transduced to express WT HLA-A2 or tailless HLA-A2. Cells were observed by flow cytometry at different time points following staining with mAb BB7.2. All experiments were repeated at least twice with similar results.

Figure 3.7

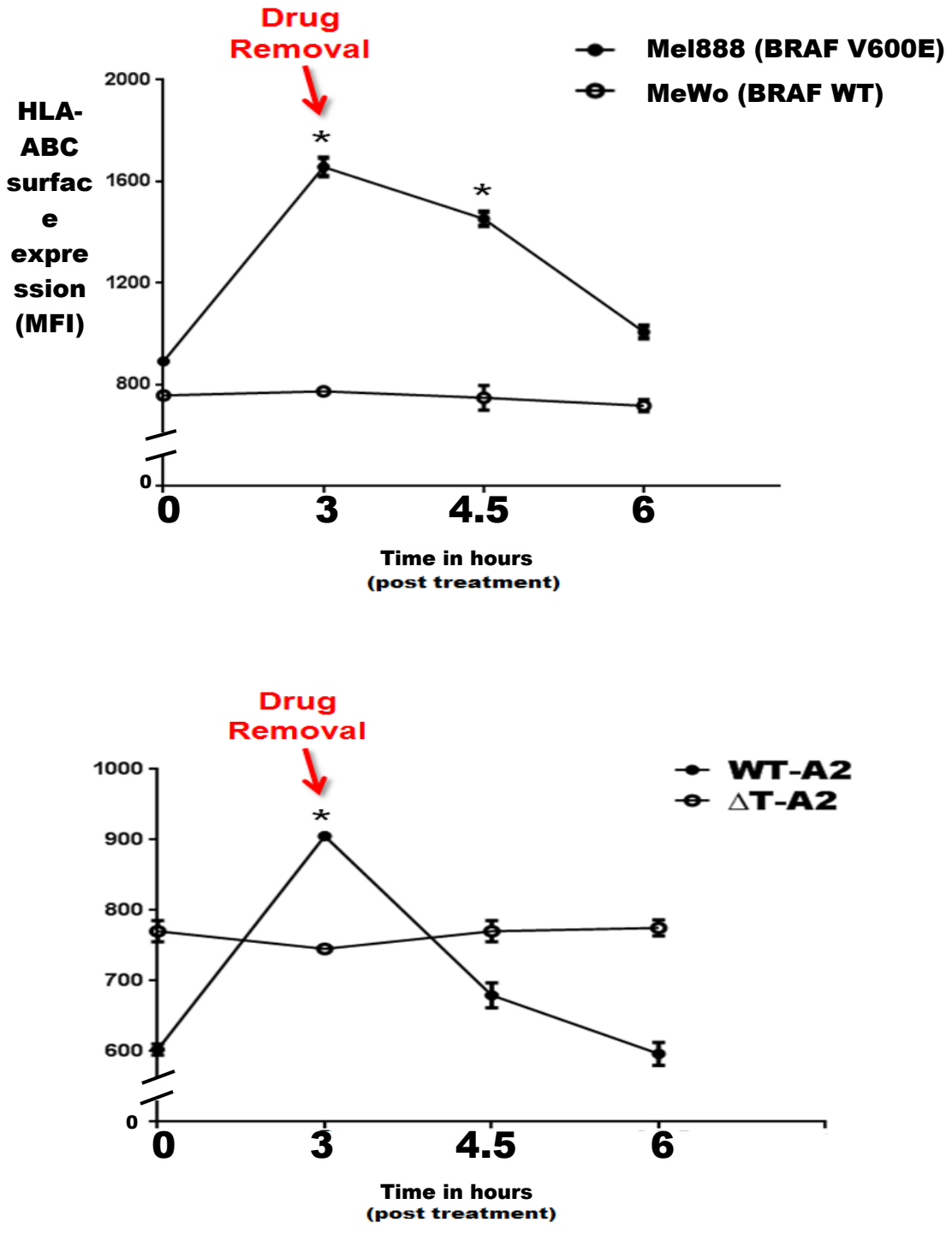

Drug Removal
Mel888 (BRAF V600E)
MeWo (BRAF WT)
HLA-ABC surface expression (MFI)
2000
1600
1200
800
0
*
*
0
3
4.5
6
Time in hours (post treatment)
Drug Removal
WT-A2
△T-A2
1000
900
800
700
600
0
*
0
3
4.5
6
Time in hours (post treatment)

Figure 3.8 MHC-I internalization in BRAF mutant cells is reduced after BRAF inhibitor treatment.

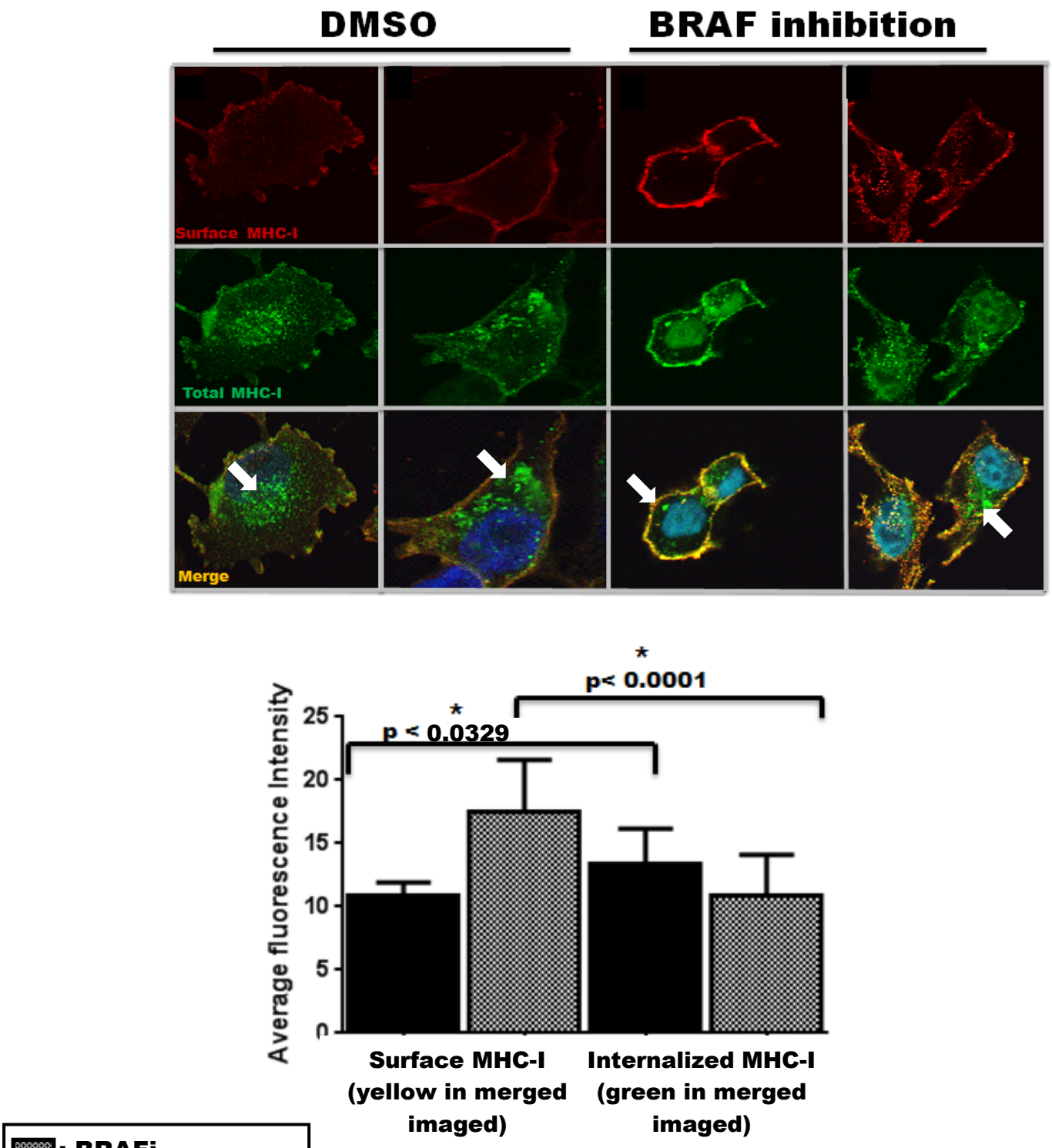
In our next set of experiments, we sought to determine the intracellular distribution of MHC-I following MAPK pathway inhibition. The BRAF mutant cell line WM793 was treated with either DMSO or the BRAFi (dabrafenib) for 2 hours, after which time the surface MHC-I was labeled with anti-mouse conjugated with W6/32 and the cells were then placed at 37 degrees for another a further 90 minutes in the presence of DMSO or BRAFi. After 90 minutes, the cells were stained for remaining surface MHC-I using goat anti-mouse IgG conjugated to W6/32. The results revealed that the DMSO-treated control cells had less MHC-I remaining on the cell surface after 90 minutes, compared with the BRAFi-treated cells, which demonstrated significantly brighter MHC-I surface expression after the same incubation time (**Fig. 3.8A**). As shown in the bottom panel of Figure 3.8, a merged image of total MHC-I (green) and the surface MHC-I (red) is displayed as a yellow color. The DMSO-treated cell line has less surface class I and more internalized MHC-I. By contrast, the BRAF-treated cell line has less internalized MHC-I and more surface class I on the cell surface because of the BRAF treatment.

To quantify the changes in intracellular MHC-I distribution, the program Image J was used to analyze cells and obtain the average fluorescence pixel intensity of each color per cell (**Fig. 3.8B**). The results of the quantification show significant differences in MHC-I intracellular distribution following BRAFi treatment: more MHC-I surface expression was seen in BRAFi-treated cells in comparison to the DMSO-treated cells. These results are consistent with our flow cytometry results and support the notion that MHC-I surface expression can be regulated by BRAF in melanoma cells.

Figure 3.8 MHC-I internalization in BRAF mutant cells is reduced after BRAF inhibitor treatment. (**A**) BRAF mutant cell line WM793 treated with control DMSO after a total of 3 hours, surface MHC-I (red), internalized MHC-I (green) and a merged image (yellow) indicates co-localization of green and red. **(B)** Quantification of the average pixel fluorescence per cell of surface MHC-I and internalized MHC-I. The white arrows indicate internalized class I. All experiments were repeated at least twice with similar results.

Imaging done by Dr. Zeming Chen

Figure 3.8


DMSO
BRAF inhibition
Surface MHC-I
Total MHC-I
Merge
*
p< 0.0001
*
p < 0.0329
Average fluorescence Intensity
25
20
15
10
5
0
Surface MHC-I (yellow in merged imaged)
Internalized MHC-I (green in merged imaged)
: BRAFi
: DMSO

CHAPTER IV:

DISCUSSION

4.1 Summary

The major aim of this thesis was to first determine the effect of MAPK pathway inhibitors on MHC-I surface expression. We also sought to determine the role of the MHC-I cytoplasmic tail in the MAPK pathway modulation of MHC-I surface expression and to assess any differences in T-cell recognition after MAPK inhibition. We also attempted to determine the mechanism behind the MAPK pathway regulation of MHC-I surface expression through observing the steady-state internalization of MHC-I in melanoma cells and the effects of MAPK pathway inhibitors on internalization. To address these questions, we compared BRAF mutant cell lines and BRAF WT cell lines, and also used BRAF mutant melanoma cell lines transduced to express WT-A2 and three cytoplasmic tail mutants. Our study reveals that the oncogenic MAP kinase pathway can modulate MHC-I surface expression in BRAF mutant cell lines in a cytoplasmic tail-dependent manner.

We found that MAPK-inhibitor induced modulation of MHC-I surface expression occurs in BRAF mutant cell lines but does not take place in BRAF WT melanoma cells. Our studies not only show that the oncogenic MAPK pathway modulates MHC-I surface expression in a cytoplasmic tail dependent matter, but also appears to be controlled by the highly conserved serine-335 residue encoded by exon 7 of the MHC-I gene. We showed that constitutive activation of the MAP kinase pathway in BRAF mutant cell lines leads to an internalization of MHC-I that can be reversed by inhibition of the pathway. The increase in surface MHC- I led to a subsequent increase in T-cell recognition and cytokine release. Our results point to a possible mechanism of immune system evasion by which melanoma cells with activating BRAF mutations downregulate MHC- I on the cell surface in order to escape immune recognition and killing.

4.2 Early effects of MAPK pathway inhibition in melanoma

We have shown that upregulation of MHC-I surface expression takes place following MAP kinase pathway inhibition (**Figures 3.4 and 3.5**); these results are supported by previous reports where MHC-I was also shown to increase on the cell surface in other melanoma cell lines following MAPK pathway inhibition [67,68]. However, our results differ significantly with respect to the time points that were analyzed. Both of these reports demonstrated MHC-I transcriptional upregulation at 24 to 72 hours following drug treatment, whereas we analyzed much earlier time points (30 minutes to 3 hours) following drug treatment. We showed that MHC-I upregulation happens much more quickly than previously reported, and therefore likely occurs independent of transcription. The changes observed at these early time points suggested a post-translational mechanism, which we explored further using HLA-A2 containing mutated phosphorylation sites. Although we also acknowledge that BRAF inhibition induces profound changes in gene expression that can lead to many downstream changes within the tumor cells, our analysis was performed at time points that precluded these changes. Previous analysis showed that the BRAF inhibitor can produce rapidly induced changes that affect more than just MHC-I. For example, a study by Khalili et al. [19] showed that transduction of melanocytes cells with the BRAF $^{(V600E)}$ gene induced the transcription of IL-1 alpha and beta. When the BRAF inhibitor vemurafenib was used on BRAF mutant cell lines, the level of interleukin 1 alpha and beta mRNA sharply declined in these cells within 3 to 4 hours. However, changes in protein expression were not observed until later time points.

4.3 The role of the cytoplasmic tail in melanoma MHC-I surface expression

The mechanism behind the regulation of MHC-I by the MAP kinase pathway has remained understudied and the mechanisms poorly understood. In this study, we attempted to shed more light on this mechanism by looking into the internalization of MHC-I in the context of a constitutively activated oncogenic MAP kinase pathway. We showed that the steady-state internalization of MHC-I took place very rapidly in BRAF mutant melanoma cell lines. Decreases in MHC-I cell surface expression could be detected as early as 30 minutes (**Figure 3.5**). This constitutive internalization of MHC-I was slowed in melanoma cells with the BRAFi, but in cells that do not harbor the BRAF mutation, the BRAFi had little to no effect. Our findings thus are consistent with a model whereby the rapid internalization of MHC-I is driven by the oncogenic MAP kinase signaling pathway. It would be of interest to determine the half-life of MHC-I molecules in BRAF mutant cells before and after BRAFi treatment. If MHC-I protein half-life is stabilized after inhibitor treatment in melanoma cells, this would be consistent with our results. Alternatively, BRAFi may not be affecting MHC-I molecular stability, but simply inducing an intracellular redistribution of MHC-I.

We also showed that the internalization of MHC-I molecules is linked to the MHC-I cytoplasmic tail. More specifically, the modulation appears to be is linked to the highly conserved serine-335 encoded by exon 7 of the MHC-I gene. The MHC-I cytoplasmic tail has already been shown to be involved in endosomal trafficking of MHC-I in dendritic cells. In contrast to our results, the tyrosine-320 encoded by exon 6 is responsible for this phenomenon [52]. However, since the MAP kinase pathway is a serine-threonine protein kinase signaling cascade [74], it makes sense that the serine residue would be the target of

phosphorylation in these cells and not the tyrosine. If the cytoplasmic serine phosphorylation site regulates MHC-I surface expression in melanoma cells with BRAF mutations, this raises the possibility that other oncogenic signaling pathways in other tumors may operate work in a similar matter. For example, oncogenic KRAS mutations are very common in pancreatic cancer, and constitutive MAPK activation is a hallmark of many other tumor types [15]. Regardless, we are the first to show that the serine phosphorylation site on the MHC-I cytoplasmic tail regulates MHC-I surface expression in tumor cells with constitutive MAPK pathway activation. A working model depicting the potential mechanism behind MHC-I modulation by oncogenic BRAF in melanoma cells is shown in **Figure 4.1**.

Potential mechanism for the modulation of MHC-I surface expression

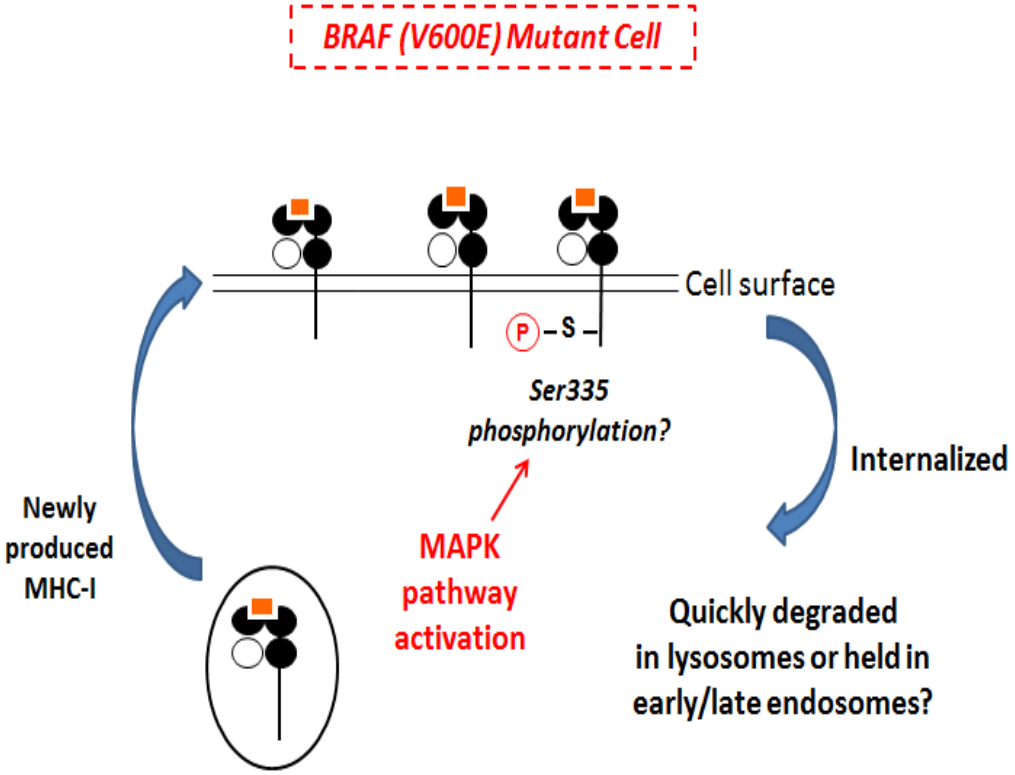


Figure 4.1 Potential mechanism for the modulation of MHC-I surface expression in BRAF mutant cell lines. Rapid internalization of newly produced MHC-I molecules may be linked to the phosphorylation of the highly conserved serine located on the MHC-I cytoplasmic tail. MHC-I may be degraded in lysosomes post internalization or held in early/late endosomes in BRAF mutant cell lines.

4.4 The role of BRAF inhibition in CD8+ T-cell recognition

MHC-I molecules play a critical role in CTL-mediated killing of tumor cells. One of the overall goals of our studies was to connect our observations of MHC-I expression and discoveries to potential improvements in CTL recognition and killing. The potential impact of MAPK pathway inhibition in T-cell recognition of melanoma tumors may have the most important implications for immunotherapy-based cancer treatment. Our results show that melanoma cells transduced with HLA-A2 point mutants behaved very differently from one another in terms of inducing T cell recognition; for example, only the WT-A2 and ΔY-A2 transduced cell lines were able to induce better T-cell recognition after BRAFi treatment. In these experiments, we showed that CTL-mediated killing can be affected even at early time points following BRAFi treatment, points and even seemingly small shifts in MHC-I expression can have significant effects on cytokine production. We observed significant changes in the amount of IFN gamma released after BRAFi treatment, but it would also be beneficial to look at the difference in the percentage of tumor cells killed following inhibitor treatment. In both the ΔT- A2 and ΔS-A2 cell lines, HLA-A2 upregulation does not take place after treatment with inhibitors at early time points **Figure 3.4**, thus implicating the cytoplasmic tail serine-335 residue in the oncogenic pathway MAPK pathway modulation of MHC-I. It is possible that in all BRAF mutant cell lines, the MHC-I molecules are being phosphorylated at this highly conserved serine, which then triggers internalization [75]. There is still a lot of information to discover about the role of the MHC-I cytoplasmic in peptide presentation and anti-tumor immunity. In DCs the removal of exon 7 encoded amino acids from the MHC-I cytoplasmic tail, which contains the serine-335, contributed to longer half-life of MHC-I, better T-cell recognition, and anti-tumor immunity [57]. However, Tyr-

320 appears to also be very important for MHC-I trafficking in APCs, suggesting that DCs and tumor cells may very well behave quite differently from one another in this regard. Serine-335 may be required in tumor cells for oncogenic induced MHC-I downregulation, but in DCs tyrosine-320 phosphorylation may be more important for a different aspect of antigen presentation. These differences show that more studies need to be conducted to better understand how the MHC-I cytoplasmic tail and MAPK signaling functions in different cell types, which could help further the development of treatments for cancer patients.

4.5 Pharmacological Implications

There are still many questions left to answer regarding confirming serine335 as a potential phosphorylation site, including identifying the exact kinase that may phosphorylate the serine residue, and whether these findings can be applied to other types of cancers with oncogenic mutations. The first step to better understand the role of the MHC-I cytoplasmic tail, is to determine the molecules involved in its function. For example, the phosphatases and kinases that use the MHC-I cytoplasmic tail as a substrate have yet to be identified. For our study, if the serine kinase that phosphorylates serine-335 in BRAF mutant cells could be identified and targeted with a highly specific kinase inhibitor it would be interesting to see if we would observe the same effects as we saw with the BRAF inhibitor. In addition to Tyr 320 playing a role in trafficking through endosomal compartments of DCs, and Serine (Ser-335) playing a role in antigen presentation in tumor cells, there is also a highly conserved ubiquitination site Lys-316 [76] that may be important for immune system recognition as well. Identifying the kinases and phosphatases for which the MHC-I cytoplasmic tail is a substrate could have significant implications for targeted therapy of cancer. If kinase

inhibitors specific for the serine or tyrosine phosphorylation sites could be discovered they could be tailored to be used in not just in melanoma treatment but also other types of cancers that have mutations in oncogenic signaling pathways that affect MHC-I expression. Our results suggest that it is possible that the oncogene-targeted treatment could be combined with immunotherapy used to increase T-cell recognition, slow tumor growth, and prevent metastasis in many types of cancers.

4.6 Future Directions

Perhaps one of the most significant takeaways from this study is the potential implications that MHC-I modulation by the MAP kinase pathway may have for antigen presentation. Peptide elutions from tumor cells have been used to discover new melanoma antigens in the past and to create vaccines [77,78,79]. It would therefore be interesting to examine the peptides eluted from the cell surface of the melanoma cells before and after MAPK inhibition. Comparisons can be made about the total number of peptides eluted from each, the diversity of the peptides, and potentially their function. It would also be interesting to study the differences in HLA-A2 restricted peptides eluted from cell lines expressing WT versus tailless HLA-A2. If differences are detected in antigen presentation, T-cells specific for those antigens could and be possibly exploited as new melanoma target antigens that would be preferentially expressed in the context of BRAFi treatment.

Further confocal microscopy studies will be required to answer all of the mechanistic questions this study has raised. We determined that MHC-I is indeed stabilized on the surface of melanoma cells following MAPK pathway inhibition but there are still more questions to be answered. For example, which intracellular what compartments do the MHC-I molecules localize to? Are they being degraded immediately, or simply being

sequestered within endocytic compartments? Confocal microscopy experiments that label the surface MHC-I molecules and chase them into the cell, along with co-staining of intracellular compartments such as early or late endosomes or lysosomes will have to be done to address these questions. It will also be interesting to characterize whether any differences are seen between trafficking the cytoplasmic tail and co-localization of the cytoplasmic tail mutants of HLA-A2.

Combination treatments involving inhibitors with other immunotherapies are currently being tested in the clinic [80,81]. Several studies, including this one, have suggested that oncogene targeted therapies and immunotherapies may show synergy [37]. Some new drugs drawing a lot of attention lately are Ipilimumab, an antibody specific for the negative regulator of T- cells CTLA- 4 [82] and molecules such as check point blockade antibodies targeting PD-L1 and PD-1 [83]. Alternative signaling pathways closely related to the MAPK pathway may also be strong candidates for combination therapy, such as the PTEN/AKT inhibitors [84]. There are many possible drug combinations to be tested and it will be interesting to determine how MHC-I surface expression and antigen presentation are affected by these combinations, and more importantly what the implications are for CTL-mediated killing of tumor cells.

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Vita

Sherille Denae Bradley was born in Mid-West City, Oklahoma on April 12, 1988, the daughter of Debra and Thomas Bradley. After completing her work at George Bush High school, in Richmond, Texas in 2006, she entered Texas State University in San Marcos, Texas. She received the degree of Bachelor of Science with a major in microbiology and a minor in biochemistry from Texas State in 2011. After graduating she entered The University of Texas Graduate School of Biomedical Sciences at Houston.

Email address:

Sherillebradley@gmail.com